Maillard-Induced Glycation of Whey Protein Hydrolysate and its Effects on Physiochemical Characteristics and Shelf-life Stability

A THESIS
SUBMITTED TO THE FACULTY OF
UNIVERSITY OF MINNESOTA
BY

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

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February 2015

Acknowledgements

First I would like to thank my advisors, Dr. Baraem (Pam) Ismail and Dr. Ted Labuza, for all their guidance, support, and insight over the past 2 years, and without whom this work could not have happened. Pam, thank you for letting me join your lab as an inexperienced undergrad 4 years ago! Your mentorship then and through grad school has helped me to grow to the scientist I am today. Ted, thank you for sharing so much of your insight and knowledge, and for helping me hone my analytical skills; I am a stronger scientist because of it. I would also like to thank my committee members, Tonya Schoenfuss and Michael Bowser, for their willingness to serve on my thesis committee, and for all the valuable insight I have gained from taking their courses.

I would also like to acknowledge the many people whose help has been invaluable over the course of this project. Thank you Qinchun Rao for all your extensive training in the lab, and for always being open to answer any of my many questions. Thank you to Courtney Lasky, for collaborating with me throughout the course of this project; I couldn't have asked for better person to work with. Thank you to Claire Boyle for so many things: for all your help at multiple steps since almost the beginning of this project, for your hard work on furosine and digestibility analysis, and for being such a positive spirit in the lab.

Of course, I wouldn't be where I am now without the support of my friends. Thank you to Lauren Gillman, Aimee Mortenson, and Jordan Walter for being awesome lab mates, classmates, and friends. Thank you to Qian Wang for your mentorship on my first undergraduate lab project that led me to where I am today. Thank you to all my friends in the Lab 122 family, and others throughout the FSCN department who have been so kind and helpful over the few couple years.

Finally, thank you to my family- Mom, Dad, and Dana- for all your unending support, and tolerance of me talking about this project and grad school for the last 2 years. And thank you to David Chau, for being there for me at every step of the way.

Dedication

This thesis is dedicated to those that inspire me to work my hardest.

To my cousins, Jessica, Jacob, and Kayla – your youthful spirit and excitement to learn is wonderful. Thank you for asking me about my work, about school, and about science. This thesis is dedicated to you, because each of you can achieve anything you set your sights on. Follow your passions and each of your will go far.

To my sister, Dana – for being supportive, thoughtful, and a worthy adversary. Your determination shows me time and time again that any goal can be met with hard-work. Thank you for being my best competitor, and keeping me on my toes. You too can achieve anything you set your sights on, and you've already shown that you can go far.

Abstract

Whey protein hydrolysates (WPH) are value-added ingredients that are experiencing a rapid increase in usage and market volume in part due to their enhanced health and functional properties. However, a challenge with the commercial use of hydrolysates in food products is their increased reactivity in many deteriorative reactions including moisture-induced protein/peptide aggregation, leading to decreased shelf-life and sensory quality. Moisture-induced protein/peptide aggregation refers to the clustering of protein molecules and the formation of aggregates, which can lead to decreased functionality, nutritional quality, and processability of protein powders. However, Maillard-induced glycation, or the covalent attachment of carbohydrates to proteins using the Maillard reaction, has been widely used to impart novel functionality to proteins via several mechanisms. It is hypothesized that Maillard-induced glycation of protein hydrolysates may reduce moisture-induced protein/peptide aggregation of these powders during storage.

The objectives of this study were twofold: (1) to produce and characterize a partially-glycated whey protein hydrolysate product using controlled and limited Maillard-induced glycation, assimilating industrial procedures where possible; and (2) to assess the ability of partially-glycated whey protein hydrolysate to retard moisture-induced aggregation during an accelerated storage study using challenging environmental conditions.

Maillard glycation of WPH was induced over 12-120 h of incubation at 60°C, 49% relative humidity (RH), and a 4:1 ratio of dextran to protein. Extent of glycation was monitored via estimation of Amadori compound formation, browning, fluorescent compound formation, free amino group loss, and visualization of protein/peptide molecular weight distribution following sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). WPH incubated with dextran for 48 h was selected for further study. Free dextran was removed from glycated and non-glycated protein and peptides with moderate success using a 2-step membrane filtration and hydrophobic interaction chromatography (HIC), yielding a final product termed ‘partially glycated whey protein hydrolysate’

(PGWPH). Basic composition of PGWPH along with a moisture sorption isotherm, digestibility and lysine blockage were determined. A controlled accelerated shelf-life study of PGWPH and WPH was then carried out at 45°C and 31%/65% relative humidity (RH) for up to 28 days to evaluate the effects of glycation on stability. Specifically, the effects of partial glycation of WPH on production of new initial-stage Maillard reaction products (MRPs), progression of MRPs generated upon production of PGWPH to intermediate and advanced stages, and moisture-induced protein/peptide aggregation were monitored. Over time, changes in color, formation of fluorescent compounds, loss of free amino groups, protein/peptide molecular weight distribution via SDS-PAGE, surface hydrophobicity, and solubility were determined. Reaction kinetics were used where possible to better understand the effects of storage conditions and sample types.

Glycation of WPH incubated with dextran was initiated within 12 h of incubation, and increased with time. Production of intermediate stage fluorescent MRPs was detected, but production of advanced stage melanoidins was minimal. PGWPH produced upon incubation for 48 h was selected for investigation in further studies, due to its moderate level of Amadori compound formation, minimal progression to intermediate and final stages of the Maillard reaction, and moderate amino group loss. The final composition of purified PGWPH was approximately 1:1 protein to carbohydrate, and displayed minimal blockage of the essential amino acid lysine (4.4%), and no significant decrease in digestibility compared to WPH. Greatest change in color, and formation of fluorescent compounds was observed for the samples stored at 65% RH, with PGWPH experiencing the most change, likely due to progression of the MRPs, generated upon production of PGWPH, to advanced stages of the reaction. Formation of insoluble aggregates and changes in surface hydrophobicity index were not detected for either PGWPH or WPH under the storage conditions studied. However, when heated at 80°C for 30 min at pH 4.5 PGWPH remained soluble while WPH lost over 60% of its solubility.

Overall, results show that partial Maillard glycation can be induced and controlled to low-levels in whey protein hydrolysate, while maintaining nutritional quality, namely digestibility and lysine availability. Results confirmed that partially-glycated whey protein hydrolysate experiences minimal deteriorative reactions during controlled storage, specifically below 65% RH and 45°C. This work is a promising step toward the advancement of protein glycation as a novel protein-enhancement technique.

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1. Literature Review

1.1 Introduction and Objectives

Whey protein is an industrial byproduct of cheese production with a market value of $9.8 billion U.S.D. in 2013 (Affertsholt 2009). Whey protein has excellent nutritional and physiological properties and displays a wide range of functionalities including solubility, gelation, foaming and emulsification (Kelly et al. 2003). The many health and functional benefits of whey protein have led to its incorporation in a variety of food products including high-protein beverages, dairy products, baked goods, confectionaries, and clinical and sports nutrition products.

Whey protein is commercially available in several forms including whey protein concentrate (WPC), whey protein isolate (WPI), and whey protein hydrolysates (WPH). Whey protein hydrolysate can be produced with many enzyme and hydrolysis conditions to yield products with enhanced functional and physiological qualities. However, a challenge with the use of protein hydrolysates is their increased molecular mobility and consequential increased chemical reactivity in degradative reactions such as Maillard browning and protein/peptide aggregation, hindering their shelf-life stability.

Moisture-induced aggregation is a challenge to the stability of high-protein powders including whey protein concentrates, isolates, and hydrolysates in particular. Moisture-induced aggregation is caused by both covalent and non-covalent interactions, and is exacerbated by protein denaturation, non-ideal storage conditions, and common interactions with other components in food systems such as lipids and reducing carbohydrates. Current solutions to the problem, such as product reformulation, use of additives, and reliance on packaging and careful storage conditions have been unsatisfactory. The potential for use of protein hydrolysates to mitigate issues of moisture-induced aggregation is promising, however, several challenges need to be addressed including increased sensitivity to environmental conditions, increased reactivity in deteriorative reactions, and decreased stability in protein systems.

Protein glycation via the Maillard reaction is a novel mechanism that may improve upon protein hydrolysates' poor stability while maintaining or enhancing their desirable nutritional and functional properties. Recent research on Maillard-induced glycation has largely been focused on the potential benefits to functional properties of food proteins glycated with various carbohydrates. However, limited information is available on how these glycated proteins behave in industrial production conditions and in real ingredient applications. Additionally, no work has of yet been completed on the potential benefits of Maillard-glycation on the stability of protein hydrolysates.

Therefore, the objectives of this research are two-fold:

1) To produce and characterize a partially-glycated whey protein hydrolysate product using controlled and limited Maillard-induced glycation, assimilating industrial procedures where possible; and

2) To assess the ability of partially-glycated whey protein hydrolysate to retard moisture-induced aggregation during an accelerated storage study using challenging environmental conditions.

1.2 Origins and Composition of Whey Proteins

1.2.1 Whey Protein Origins

Milk and milk-based products are popular ingredients in many foods due to being sources of proteins with excellent nutritional and functional properties. Milk protein is made up of two major classes: casein proteins and whey proteins, comprising 80% and 20% of total milk protein, respectively, and each differing greatly in structure, functionality, and organization within fluid milk (Hui et al. 1993).

Fluid whey is comprised of approximately 0.7-0.8% protein, 3.5-4.7% lactose, 0.05-0.1% milkfat, 0.5-0.8% ash, with the remainder being water (Page et al. 2004). Historically, whey had little value, and so was used to feed farm animals or used as field fertilizer (Caric 1993). On average, nine liters of whey is produced with every kilogram of cheese, resulting in a large amount of whey to dispose of (Tunick 2008). About 960 million pounds of dried whey were generated in the U.S. in 2013 alone (National Agricultural Statistics Service 2014). Whey has even been viewed as a detrimental byproduct of a high biological oxygen demand (BOD), and thus is environmentally costly to dispose of (Caric 1993). However, whey protein specifically has gained new interest since the 1970's when improved processing techniques made it easier to create whey products with improved quality and functional properties (Fox 2003; Tunick 2008).

The whey that is produced industrially can be categorized into two broad classes: sweet whey or acid whey. Sweet whey is the whey left from production of certain cheese, such as cheddar, Swiss and mozzarella, and has a pH of 5.5-6, and a maximal titratable acidity of 0.16% CFR (Page et al. 2004). In contrast, acid whey is whey left from other cheeses like cottage and ricotta cheese, or Greek yogurt, which are produced by direct acidification of milk. This whey has a pH of 4.4-4.6, and titratable acidity of 0.35-0.44% (Page et al. 2004). Acid whey is typically considered to be of lower value because its high lactic acid content makes it difficult to dry and process, so sweet whey is the whey more commonly used for the production of industrial ingredients, namely whey protein ingredients.

1.2.2 Whey Protein Composition

Though the composition of whey is affected by the method by which it is produced the major whey proteins are always present, and in roughly the same proportion (Table 1). The major whey proteins are β-lactoglobulin, α-lactalbumin, bovine serum albumin, and immunoglobulins, while minor proteins include a variety of important enzymes and other proteins such as lactoperoxidase and lactoferrin (Yiu H. Hui 1993; Walstra et al. 2006). Sweet whey protein may additionally contain glycomacropeptide and proteose peptone, which originate from the cheese-making process, upon enzymatic hydrolysis of κ-casein and β-casein, respectively (Fox 2003).

Table 1. Major whey protein components: Adapted from Advanced Dairy Chemistry Vol. 1 (Fox 2003)

Protein	% of total whey protein
β-lactoglobulin	50%
α-lactalbumin	20%
Serum albumin	6%
Immunoglobulins	12%
Proteose peptone	12%

β-lactoglobulin is the primary protein component, constituting more than 50% of whey protein (Fox 2003). β-lactoglobulin exists in three main genetic variants, A, B, and C, which differ slightly in their primary structure (Fox 2003). β-lactoglobulin has a molecular weight of approximately 18 kDa and is comprised of 162 amino acids, a relatively high number of those being sulfur-containing amino acids (Fox 2003). The presence of two intramolecular disulfide bonds greatly impacts the tertiary structure of β-lactoglobulin as these covalent cross-links stabilize the protein's compact globular structure (Walstra et al. 2006). The molecule has an additional free thiol group in its interior moiety, which gets exposed upon denaturation and consequently contributes to intermolecular association and polymerization (Mulvihill & Donovan 1987). In general, like the rest of the whey proteins, β-lactoglobulin has a highly ordered secondary structure,

comprised of 43% β-sheets, 10-15% α-helices, with the remainder being random coil (Fox 2003). At physiological pH (pH 5.5-7.5), β-lactoglobulin forms dimers stabilized mostly by hydrophobic interactions (Walstra et al. 2006). At pH 3.5-5.5, due to the low molecular net charge and lack of electrostatic repulsions near its isoelectric point, the dimers of β-lactoglobulin associate to form octamers (Walstra et al. 2006). However, below pH 3.5 or above 7.5, intermolecular repulsions cause β-lactoglobulin dimers to dissociate into monomers (Walstra et al. 2006). The physiological function of β-lactoglobulin is still not well understood. While β-lactoglobulin is not a protein intended for neonate nutrition, as it is fairly resistant to hydrolysis, it may have a role in transporting Vitamin A, and in stimulating lipase activity (Fox 2003).

The whey protein present in the next highest concentration is α-lactalbumin, making up approximately 20% of whey proteins (Fox 2003). Milk can contain either of the two genetic variants of α-lactalbumin, A and B, which differ slightly in primary structure (Fox 2003). One molecule of α-lactalbumin has a molecular mass of 14 kDa, and is comprised of 123 amino acids (Fox 2003). It is less ordered than β-lactoglobulin with 26% α-helices, 14% β-sheets, and the remaining being random coil (Fox 2003). The tertiary structure of α-lactalbumin is stabilized by its four intramolecular disulfide bonds, and it typically binds one residue of divalent calcium (Ca^{2+}), which lends the protein substantial heat stability compared to the other whey proteins (Walstra et al. 2006). Similar to β-lactoglobulin, individual α-lactalbumin molecules do associate, but the structures formed are not as well understood (Walstra et al. 2006). Large aggregates may form at pH slightly lower than α-lactalbumin's isoelectric point (Kronman et al. 1964). α-lactalbumin serves an important role in milk, where it is a coenzyme required in the biosynthesis of lactose by the enzyme lactose synthase (Walstra et al. 2006).

The other whey proteins are present in much lower concentrations, and so have lesser impacts on the overall whey protein functionality. Serum albumin is much larger than either α-lactalbumin or β-lactoglobulin, with 582 amino acids and a total molecular mass of 66 kDa (Fox 2003). The protein is stabilized by 17 disulfide bonds, and contains an additional free thiol group (Fox 2003). Serum albumin has necessary functions in the

serum of blood, where it originates from, but has little functional or physiochemical properties in milk (Fox 2003). Milk free of colostrum contains low concentrations of three classes of immunoglobulins, abbreviated IgA, IgG, and IgM. The purpose of all three is to provide immunity to calves and to protect the mammary glands of the cow from disease (Hurley 2003). However, these are often denatured and inactivated by heat treatment of milk and milk products (Walstra et al. 2006). Various peptide fractions of casein may also be present in fluid whey, including N-terminal segments of β-casein, previously referred to as proteose peptone, and the cleaved glycomacropeptide of κ-casein, solubilized by the action of rennet.

1.3 Nutritional Quality and Health Benefits of Whey Protein

Many connections have been made between consumption of low-fat dairy foods and health. Diets rich in dairy products have been associated with decreases in blood pressure (Engberink et al. 2009; Toledo et al. 2009; van Meijl & Mensink 2011), type 2 diabetes (Elwood et al. 2008, 2010), colon cancer (Elwood et al. 2008; Pufulete 2008; Huncharek et al. 2009), stroke and heart disease (Elwood et al. 2008, 2010). Consumption of dairy products has also been associated with decreased levels of inflammation (Esmaillzadeh & Azadbakht 2010; van Meijl 2010) and decreased development of the metabolic syndrome (Pereira et al. 2002; van Meijl et al. 2008). Considering all these potential benefits, it is not surprising that consumption of whey protein has also been linked to good health.

Dairy products make up approximately 30% of the dietary protein of North American and European diets (Fox 2003); this is in part due to their reputation as versatile and high-quality sources of protein. Highly digestible and containing all the essential amino acids in the relative proportions required for health, whey protein is considered to have excellent protein quality. Based on the protein digestibility corrected amino acid score (PDCAAS) system of evaluating protein quality, whey protein has the maximum score of 100, making it on par with other high-quality animal proteins (Walstra et al. 2006). The protein efficiency ratio (PER) is another system used to evaluate protein quality using

the ratio of weight gain in growing animals to protein consumption. The PER of whey protein is 3.2, which is higher than all other protein sources except egg protein (Walstra et al. 2006).

Dietary protein is known to contribute to satiety, even more than consumption of fat and carbohydrate (Halton & Hu 2004). Consumption of whey protein, specifically, before meals has been correlated with decreased food (Anderson & Moore 2004), and reduced post-meal blood glucose and enhanced insulin secretion (Horowitz et al. 1993). Reduction in post-meal blood glucose was attributed to the protein's effect on gastric emptying and its concentration of certain amino acids such as isoleucine (Dangin et al. 2002; Luhovyy et al 2007; Veldhorst et al. 2008). Researchers have observed faster digestion and hormonal response to consumption of whey protein compared to other proteins such as casein, as its structure and solubility allow it to be more easily metabolized, and its constituent amino acids consequently absorbed faster (Boirie et al. 1997). Whey proteins have been found to reduce short-term appetite better than caseins (Hall et al. 2003). Whey protein supplementation led to increased detection of cholecystokinin (CCK), a hormone associated with satiation, in the plasma (Hall et al. 2003).

Whey protein has become the protein supplement of choice for athletes and individuals hoping to build more muscle for a multitude of reasons. Whey protein reaches the jejunum of the small intestine very quickly and largely intact (Anderson & Moore 2004), and is digested over a longer length of the intestine than most proteins (Boirie et al. 1997; Newport and Henschel 1985; Miranda and Pelissier 1983). Whey protein digestion leads to rapid and steep increase in plasma amino acids (Boirie et al. 1997), which is desirable for post-exercise muscle repair and synthesis. Additionally, researchers have found that postprandial protein synthesis is stimulated with whey protein consumption (Boirie et al. 1997). This is believed to be in part due to whey protein's high content of the amino acid leucine, a branched-chain amino acid, which plays a role in stimulating protein synthesis (Anderson & Moore 2004; Yudkoff et al. 2005).

Whey protein has garnered much positive attention and use in protein-supplementation products due to being the highest natural food source of branched-chain

amino acids (Page et al. 2004). The branched-chain amino acids: isoleucine, leucine, and valine, are important for muscle endurance, power, and repair (Page et al. 2004). As 'essential' amino acids, a sufficient supply of these three amino acids must come from the diet. Approximately one-third of muscle is made up of these three amino acids, and so consumption of these amino acids is vital for muscle synthesis (Page et al. 2004). Additionally, these amino acids can be used as an energy source for skeletal muscle during endurance exercises. Branched chain amino acids can be directly taken up by muscles during exercise, unlike the other amino acids which must first be processed by the liver (Page et al. 2004).

Whey protein has been widely recognized for its excellent nutritional properties and its many potential contributions to good health, however, these benefits are of little use without a proper delivery system. For that reason much focus has also been placed on efficient production of whey protein ingredients, and on understanding their functionality in various food systems.

1.4 Whey Protein Ingredients Production and Application

1.4.1 Production of Whey Ingredients

Production of dried milk powders has been used for centuries to remove water from milk as a means to decrease weight and volume of the product and as a preservative measure, thereby making transportation and storage more economical (Caric 1993). The production of dried milk powders without the need for additives such as sucrose or sodium carbonate started in the end of the 19th century, though dried whey powders were not widely produced until the late 20th century (Caric 1993; Tunick 2008).

Whey left from production of cheese, yogurts, or other dairy products can be processed into many whey ingredients which differ in composition and so differ in intended application. Whey can also be voluntarily graded for quality by the USDA. The distinction of the USDA U.S. Extra Grade is given to whey with appropriate flavor, physical appearance, microbial characteristics, milkfat and moisture contents (AMS 2000).

Dried whey powders can be produced using a variety of common unit operations. Depending on the source of the fluid whey, it is often centrifuged or filtered first to remove cheese fines, clarified to remove any remaining milk fat, and then pasteurized to eliminate pathogens, spoilage organisms, and any remaining cultures used in cheese production (Walstra et al. 2006). Fluid whey is then concentrated to remove much of the water and increase the total solids content to 40% or more (Caric 1993). This is done using evaporation, reverse osmosis, or both in tandem (Kelly et al. 2003).

The most basic dried whey ingredient is often referred to as 'dry sweet whey', and is of the same composition as fluid whey but has had almost all of the water removed (Page et al. 2004). The approximate composition of dry sweet whey is 10.5% protein, 71% lactose, 9% minerals, and 1% fat (Walstra et al. 2006). Dry sweet whey is used in products in which both the protein and the significant amount of lactose is beneficial, such as bakery products, frozen desserts, snack foods, and dry mixes for gravies and soups (Page et al. 2004).

Lactose and minerals can be removed from the fluid whey, and the product may be further concentrated to produce higher-value whey ingredients. Lactose is removed for use in other applications and to decrease the hygroscopicity of the final whey powder, and is separated out by crystallization, filtration or chromatographic techniques (Caric 1993). Minerals are typically removed to prevent defects in taste or interference in some food applications, and are separated out by electrodialysis or ion-exchange chromatography (Caric 1993). The last step of the process is drying the remaining material to approximately 4% moisture using spray, roller, or fluid bed drying, each process producing products of different quality and value (Caric 1993). Dried whey powders are also typically instantized, causing the dried powder to form agglomerates with improved wettability and dissolving characteristics (Caric 1993).

The most common separation technologies used in modern production of high-protein whey ingredients are ion-exchange chromatography and ultrafiltration. Ultrafiltration of fluid whey can be used to remove low molecular weight compounds such as minerals, lactose, and vitamins, while concentrating and increasing the protein content

at the same time (Caric 1993). Ultrafiltration can be paired with diafiltration to further produce whey products with protein contents of up to 95% (Caric 1993). Ion-exchange chromatography makes use of charged resins to purify whey proteins from lactose, minerals, and other undesired components, and can be used to achieve whey protein isolates with greater than 95% protein (Page et al. 2004).

When portions of the mineral and lactose are removed via filtration the resulting ingredients are then referred to as 'whey protein concentrates'. Whey protein concentrates (WPC) contain at least 25% protein on a dry basis, and are divided into various categories based on their protein content such as 'WPC34' with 34% protein and 'WPC80' with 80% protein (Page et al. 2004; Tunick 2008). As the composition of products given the designation of whey protein concentrate are so varied, the possible applications of these products also vary greatly. WPC34 ingredients are often used in bakery mixes, and as dairy product stabilizers (Page et al. 2004). In contrast, WPC80 ingredients are frequently seen in soups, nutritional beverages, and meat products (Page et al. 2004).

The most purified whey protein ingredient is whey protein isolate, produced either using membrane filtration or ion-exchange chromatography. Whey protein isolate (WPI) contains at least 90% protein and typically has less than 1% lactose, fat, and less than 2% minerals (Page et al. 2004). This ingredient can be used in a wide variety of products for its functionality or nutritional properties, but is most notably used in formulations for nutritional supplement bars and beverages, and is often packaged for direct purchase by consumers for at-home protein supplementation (Page et al. 2004).

1.4.2 Functionality and Application of Whey Protein Ingredients in Foods

As noted previously whey protein ingredients can be used in a variety of products to impart desired functional, organoleptic, and textural characteristics with the nutritional benefit of added protein. Whey protein has a clean flavor and have better functionality than most plant proteins (Damodaran 1994). The functionality of whey protein ingredients, however, is highly dependent on a variety of factors including environmental factors (pH, humidity), and processing techniques or modifications like thermal or chemical treatments.

The most important functional characteristics of whey protein ingredients are solubility, water-binding, gelation, emulsification, foaming, and whipping (Kelly et al. 2003).

Whey protein is highly soluble at nearly any pH, even around its isoelectric point (pH 4.5-5.5), a unique functional property attributed to the protein's structure (Mulvihill & Ennis 2003). Whey protein naturally has high surface hydrophillicity, contributing to high intermolecular repulsions and improved solvation (Fox 2003; Moro et al. 2013). Being globular in structure, whey protein folds in a way that buries most of the hydrophobic amino acids within its interior moiety to reduce thermodynamically unfavorable interactions with water (Damodaran 1994). This excellent solubility makes whey protein ideal for use in high-protein beverages, medical nutrition supplements, powdered coffee creamers, and soups and sauces (Kilara 1994). The solubility of whey protein is maintained even through moderate heat treatment at 95°C if at pH less than 3.5 or greater than 6, however, its solubility is greatly decreased by heating above 70°C around its isoelectric point. Under these conditions the protein unfolds, exposing the hydrophobic interior and free thiol group, leading to protein aggregation and precipitation due to reduced intermolecular repulsions, increased hydrophobic interactions, and formation of disulfide bridges. Nevertheless, it is still considered one of the most soluble sources of dietary protein (Damodaran 1994).

While whey protein's solubility may decrease upon heating, its thermal lability often allows for excellent gelation properties when environmental conditions are controlled (such as heating rate, temperature, ionic strength and pH). Upon thermal denaturation of whey protein and exposure of its hydrophobic interior, and in the case of β-lactoglobulin, its reactive free thiol group (H. Singh & Havea 2003), intermolecular hydrophobic and disulfide interactions take place, allowing for gel network formation (H. Singh & Havea 2003). The unfolding of whey protein upon thermal denaturation also enhances the protein's water-binding capacity by creating more available binding sites for water (Kilara 1994). The ability of whey proteins to bind-water and gel is ideal for improving yield, texture, viscosity, and stability of various dairy products like ricotta, cream cheese, and yogurt (Page et al. 2004). Its water-binding can also increase the viscosity and improve

the mouth-feel of fluid products like soups, sauces, and creamy beverages (Kilara 1994). The use of whey proteins for water-binding and gelation is also important to the structure of imitation fish and seafood products, and luncheon meat products (Page et al. 2004). Moreover, gelation and water binding are important for the structure of doughs and retention of moistness in baked goods (Page et al. 2004).

The gels formed by whey protein with heat treatment are non-reversible, as both non-covalent and covalent interactions are disrupted during denaturation and as intermolecular non-covalent and covalent interactions occur during gel network formation (Damodaran 1994). The firmness, strength, and opacity of whey protein gels can be altered by varying the pH and ionic strength. Conditions resulting in reduced solubility such as pH near the isoelectric point of whey protein or high ionic strength lead to opaque gels (Z Y Ju & Kilara 1998). Strong, firm gels can be created by increasing the heating to just above the denaturation temperature of whey proteins, and by following a moderate heating rate to avoid rapid aggregation and precipitation (Damodaran 1994).

While whey protein in its native form lacks the ideal balance of hydrophobic and hydrophilic regions on the surface that makes a molecule ideal for emulsification, whey protein can unfold at the interface of emulsions and lend adequate emulsification characteristics for a variety of applications (Page et al. 2004). Heat-treated and partially denatured whey protein can be used and potentially replace egg ingredients in various desserts, candies, sauces, and soups (Kilara 1994). Moderate thermal denaturation causes unfolding that renders whey protein more amphipathic (Morr 1979; Voutsinas et al. 1983). Whey protein may also be used to stabilize foams (Kilara 1994). Whey protein is useful in whipping applications due to its ability to quickly diffuse to the air-water interface and unfold to stabilize the foam, and thus may serve as a replacement for eggs in candies and ice cream (Mangino et al. 1987; Page et al. 2004).

The net effects of whey protein's chemical characteristics and of controlled environmental conditions allow for excellent performance in a wide variety of applications. This, along with whey protein's reputation as a nutritional powerhouse has led to considerable economic value of the former waste-stream.

1.5 Economic Significance of Whey Protein Ingredients

The use of whey protein ingredients and products has been on the rise in recent years and is poised for future growth. In 2013, over 85 million pounds of WPI and over 480 million pounds of WPC were produced, up 34% and 13.4% from 2012, respectively (National Agricultural Statistics Service 2014). Global production of whey protein products had an estimated market value of 9.8 billion U.S.D. in 2013, and is forecasted to reach 11.7 billion U.S.D. by 2017 (UBIC Consulting 2014). Favorable consumer perceptions of whey protein and other proteins have helped boost their perceived value and use in products. More than half of the adults in the U.S. want more protein in their diet, driven by health and wellness trends and by protein's associations with satiety, muscle-building, and healthy aging (The NPD Group 2014). In 2013, nearly 25% of adults in the U.S. reported that when they check the nutrition facts panel for a product they're looking for the protein content (The NPD Group 2014). And half of American consumers are flexible protein users, and are looking to non-meat sources to meet their protein needs (The NPD Group 2014). Whey proteins are well suited to meet the needs of flexible protein users, particularly in the growing performance food and drinks category, estimated to be worth more than 10 billion U.S.D. (Mintel 2013), as they have become widely available, and display excellent nutritional and functional quality.

Whey protein is an excellent functional food ingredient due to its wide availability, excellent functionality, and excellent nutritional properties, which has led to its incorporation in a wide variety of food products. These same qualities, along with favorable consumer perception, make whey an excellent candidate for the production of value-added food ingredients such as whey protein hydrolysates.

1.6 Usage of Whey Protein Hydrolysate Ingredients

In addition to traditional protein isolate and concentrate products, many proteins are also available as hydrolysates which have undergone varying degrees of hydrolysis to produce value-added products with improved health, functional, or other properties. Protein hydrolysates are used most notably in the formulation of infant formulas, sports

nutrition products, clinical nutrition products, and other functional foods or dietary supplements (Affertsholt 2010). Currently protein hydrolysate ingredients make up a fraction of the market volume of their isolate and concentrate counterparts, but their market growth greatly outpaces non-hydrolyzed protein ingredients (Affertsholt 2009). Global production of dairy hydrolysates exceeded 20,000 metric tons in 2010, with a market value exceeding 300 million U.S.D, and a projected short-term yearly growth of 8-10% (Affertsholt 2010). More specifically, global production of whey protein hydrolysates is projected to have an annual average growth rate of 3.4% from 2008-2018 (Frost & Sullivan 2013).

1.6.1 Production of Whey Protein Hydrolysate Ingredients

Hydrolysis is one of many methods available for altering a protein's structure. Hydrolysis disrupts the primary structure of a protein by breaking the peptide bonds, resulting in the production of peptides with lower molecular weight than the native protein. Protein hydrolysis is primarily accomplished through chemical or enzymatic means (Alder-Nissen 1986). Chemical hydrolysis uses strong acid or base to hydrolyze the peptide bonds of proteins, and the resulting peptides are heterogeneous, as acids and bases hydrolyze non-specifically (Alder-Nissen 1986). More common is the use of enzymes to hydrolyze proteins, as specific enzymes can be used that target select sequences of the protein, resulting in a more uniform and consistent hydrolysate (Alder-Nissen 1986; Lahl & Braun 1994). Use of enzymatic methods also eliminates the adverse side-reactions that may occur with chemical hydrolysis, including a decrease in nutrition quality by destruction of certain amino acids such as tryptophan, and through the formation of carcinogenic byproducts such as lysinoalanine (Lahl & Braun 1994).

Properties of hydrolysates are affected by many factors both intrinsic and extrinsic. Factors that affect protein hydrolysate characteristics include the type and specificity of the enzyme used, treatment and purity of the protein before hydrolysis, the physiochemical conditions during hydrolysis (including pH, temperature, ionic strength, and presence or absence of activators or inhibitors), the extent or degree of hydrolysis (DH), the technique

used for enzyme inactivation, and any post-hydrolysis treatment, such as drying (Alder-Nissen 1986).

In the production of WPH, specifically, a number of enzymes have been used with success. The enzyme chosen often is dictated by the desired application of the hydrolysate. Digestive enzymes are commonly used for the production of WPH including pepsin, trypsin and chymotrypsin (Ju et al. 1995; Groleau et al. 2003; Konrad et al. 2005; Hernandex-Ledesma et al. 2005; Pouliot et al. 2009). But plant enzymes have also been used such as papain and bromelin (Nakamura et al. 1993); as well as bacterial proteases such as that of *Bacillus licheniformis* (Madsen et al. 1997; Creusot et al. 2006). Often times a mixture of enzymes are used to achieve desired effects in functionality or nutrition, and so commercial enzyme preparations are designed with more than one enzyme to target a specific application (Alder-Nissen 1986). One such commonly used commercial enzyme preparation is Alcalase® (Novozymes, Bagsværd, Denmark) which is a mixture of enzymes derived from *Bacillus licheniformis,* and which has been observed to generate whey protein hydrolysates with enhanced gel-forming properties (Spellman et al. 2005; Creusot and Gruppen 2008).

Whey protein hydrolysates are produced on an industrial scale in a few ways. Traditional production of protein hydrolysates is a batch process (Alder-Nissen 1986). In batch production the protein substrate is added to a vat along with the enzyme and the reaction is monitored until the desired DH is achieved, at which point the enzyme is inactivated with heat or pH (Alder-Nissen 1986). More recently, semipermeable membrane enzymatic reactors have gained interest. Production of hydrolysates using membrane enzymatic reactors is similar to batch production except that the vat has a semi-permeable membrane that retains the enzyme along with proteins and peptides above a certain size, and allows smaller compounds to pass through (Guadix et al. 2006; Cheison et al. 2007a, 2007b). This membrane allows for selective hydrolysis of proteins until they reach a certain size, pass through the filter and are not hydrolyzed any further (Cheison et al. 2007a, 2007b). This allows for cost-effective re-use of enzyme, reproducible generation

of peptides of a specific and controlled length, and allows for high enzyme concentrations and continuous production (Cheison et al. 2007a, 2007b).

The environmental conditions during hydrolysis have a significant impact on the efficiency of the process, and the final DH, quality and functionality of the resulting hydrolysates. The DH of whey protein hydrolysates is maximized with the use of the optimal temperature and pH of the enzyme(s) used for hydrolysis. However, whey protein hydrolysates with high DH currently have only a few major uses, specifically reduced-allergencity formulas for infants with milk allergies and medical diets, due to the poor functionality of high DH hydrolysates. Instead, DH is often limited to low or moderate levels (~5-10%) to enhance functionality, including solubility (Mullally et al. 1994), emulsification (Turgeon et al. 1991), emulsification (Kuehler & Stine 1974), and gelation (Creusot & Gruppen 2007).

Post hydrolysis treatments of whey protein hydrolysate may include dialysis, fractionation, de-bittering, and drying (Ismail & Gu 2010). As enzymes are sensitive to pH and perform best at their optimum pH, the environmental pH during hydrolysis is typically monitored and adjusted using acid or base. However, pH adjustment using acid and base results in the generation of salts, which are often removed using dialysis to maintain the hydrolysate purity (Alder-Nissen 1986). In the production of whey protein hydrolysate with composition targeted for particular usage, such as selecting for particularly bioactive peptides or peptides with high aggregation ability, fractionation using isoelectric focusing, nano-filtration or chromatography have been used to retain only peptides with the desired characteristics (Ting et al. 2007; Saint-Sauveur et al. 2008). If bitter peptides are generated during hydrolysis, the hydrolysates may be de-bittered using a variety of techniques including activated carbon treatment (Cheison et al. 2007a). The final post-hydrolysis treatment is drying, which is typically carried out using roller drying or spray drying (Alder-Nissen 1986).

1.6.2 Health Benefits of Whey Protein Hydrolysate Ingredients

With the rising consumer trends towards health, wellness and functional foods, one of the most enticing reasons for the production of protein hydrolysates is the potential benefits to consumer health and nutrition. The potential health benefits of protein hydrolysate consumption have been widely studied and include the generation of bioactive peptides, increases in digestibility, and decreases in allergenicity.

One of the most consequential benefits of the production of protein hydrolysates is the generation of bioactive peptides. Bioactive peptides are specific sequences of amino acids that can be liberated from a protein upon hydrolysis and are associated with positive health benefits such as antihypertensive, anti-inflammatory, antimicrobial, antioxidative, or opioid activity, among other physiological benefits. Bioactive peptides are typically short in length, often 2-9 amino acids long (Kitts & Weiler 2003); however, many bioactive peptides are produced with only limited degree of hydrolysis (2-8%) (Ismail & Gu 2010) allowing for retention of much of the original protein's functional properties.

Many bioactive peptides have been identified in or produced from whey protein. Peptides with opioid activity called lactorophins have been derived from both β-lactoglobulin and α-lactalbumin (FitzGerald & Meisel 2003). Whey protein peptides called lactokinins derived from β-lactoglobulin, α-lactalbumin and bovine serum albumin have been shown to combat increases in blood pressure by inhibiting the angiotensin-I-converting enzyme (ACE), which increases blood pressure via the renin-angiotensin system (Meisel 1997; FitzGerald & Meisel 2003). Peptides from lactoferrin have been reported to have potent antimicrobial activity (FitzGerald & Meisel 2003). Also, whey protein hydrolysates with low DH have been observed to have antioxidant properties (Dryáková et al. 2010; Peña-Ramos et al. 2004). The use of bioactive peptides to improve health and combat illness is promising as bioactive peptides are naturally occurring, non-toxic, with minimal if any side-effects or dosage effects (Danquah & Agyei 2012). These characteristics make bioactive peptides a persuasive replacement for many of the synthetic compounds currently used.

Whey protein hydrolysates also serve as valuable ingredients for products such as infant formula or clinical diet formations. Hydrolysis increases the digestibility of whey proteins (Sinha et al. 2007). Additionally peptides are more bioavailable than either intact proteins or free amino acids (Danquah & Agyei 2012). Extensively hydrolyzed whey protein hydrolysates are often used in the formulation of many medical diets for illnesses such as liver disease, Chron's disease, pancreatitis, and ulcerative colitis (Clemente 2001; Poteir and Tome 2008). Moreover, disruption of the primary structure of proteins upon hydrolysis may result in the destruction of linear or conformational epitopes, hence decreasing the allergenicity of a protein. Extensively hydrolyzed whey protein has been successfully used as a replacement for traditional formulas for infants sensitive or allergic to cow's milk and soy (Merritt et al. 1990; Oldaeus et al. 1992; Nakamura et al. 1993; Guadix et al. 2006).

Many health benefits may arise from the production of whey protein hydrolysates, however excess hydrolysis may be associated with a decrease in functionality. It is important that hydrolysis conditions are designed to optimize both the health and functional benefits of whey protein hydrolysates.

1.6.3 Functional Benefits and Usage of Whey Protein Hydrolysate Ingredients

Whey protein hydrolysates can display certain functional benefits depending on their protein/peptide profile and the DH. Changes in protein size and primary structure lead to changes in the interactions between proteins and peptides, and alter the effects of environmental factors, ultimately leading to changes in the protein's functionality. Most of the changes in functionality of protein hydrolysates are due to the net decrease in molecular weight, and the exposure of hydrophobic and ionic groups (Ismail & Gu 2010). Whey protein hydrolysates may display improved solubility, lower viscosity, improved emulsification and foaming capacity, improved gelation properties, and improved thermal stability.

Protein hydrolysates with improved solubility and reduced viscosity, allow for the formulation of high-protein beverages with improved stability (Mullally et al. 1994). Though the solubility of native whey protein is excellent, high heat-treatment can cause severe loss in solubility (Wang & Ismail 2012). Hydrolysis has been shown to improve the solubility of heat denatured whey protein even around the isoelectric point by increasing the number of accessible charged carboxyl and amine groups (Perea et al. 1993; Mutilangi et al. 1996).

Whey protein hydrolysates with low DH may also display enhanced surface activity, as the smaller peptides are more readily adsorbed into the interface and may display greater molecular flexibility and thus faster unfolding at the interface than their native non-hydrolyzed counterparts (Damodaran 1994). These qualities along with other structural factors such as amphipathic character, hydrophobicity, and net charge may make particular whey protein hydrolysates more suited for specific emulsification or foaming applications (Turgeon et al. 1991; Ismail & Gu 2010).

Singh and Dalgleish (1998) observed maximal emulsifying capacity using WPH with DH of 10-20%, however emulsion stability was poorer compared to native proteins unless a lower DH was used (Singh and Dalgleish 1998; van der Ven et al. 2001; Foegeding et al. 2002). Whey protein hydrolysates with low DH (2-5%) displayed improved emulsion and foaming capacity and stability (Althouse, Dinakar, and Kilara 1995; Kuehler and Stine 1974; Foegeding et al. 2002).

Whey protein hydrolysates that were extensively hydrolyzed displayed poorer gelation properties compared to their native proteins, as shorter peptide chains can make network formation more difficult (Huang et al. 1999). However, controlled and limited hydrolysis using specific enzymes may improve gelation properties of food proteins, including whey protein. Whey protein gelation can be improved when hydrolysis exposes hydrophobic residues, allowing for intermolecular hydrophobic interactions (Creusot & Gruppen 2007). For instance, hydrolysis of whey protein using proteases from *Bacillus licheniformis* have resulted in improved gel formation (Otte et al. 1996; Creusot et al. 2006; Creusot and Gruppen 2007).

Several researchers have also reported that the hydrolysis of whey protein leads to changes in gelation properties. For instance, acid-set gels made from non-hydrolyzed whey protein are typically elastic, while gels made from chymotrypsin hydrolyzed whey protein have reduced elasticity making them better suited for certain food applications like yogurt (Rabiey & Britten 2009).

Sometimes gelation is undesirable, such as in high-protein beverages requiring heat-treatment. In these cases extensive hydrolysis leading to poor gel network formation is preferred for its improved thermal stability. Hydrolysis has been used as a method to impart heat stability of whey protein, because hydrolysis disrupts the protein's secondary structure leading to less secondary structure loss during subsequent heating (Foegeding et al. 2002). However, extensive hydrolysis has detrimental effects on flavor because of the excessive production of bitter peptides (Murray & Baker 1952).

A variety of whey protein hydrolysates have been produced in laboratory research yielding a diverse array of biological and functional benefits, however a caveat of these results is that many challenges that would be faced in industrial production are not addressed. If whey protein hydrolysates are to have greater market value, and increased inclusion in products these challenges must be addressed.

1.6.4 Challenges with the Usage of Whey Protein Hydrolysate Ingredients

Though WPH have many benefits to their use, they also present several challenges, one of which is the perception of bitter flavor. Protein hydrolysates, particularly those which are extensively hydrolyzed, have a perceivable amount of bitter peptides, which negatively affect the flavor profile of the final products (Murray & Baker 1952). Particularly, peptides that have a higher number of hydrophobic amino acids have been associated with the bitter taste (Matoba & Hata 1972). Although the perception of bitter peptides has no impact on the functional or nutrition properties of WPH, it drastically decreases their sensory quality and hence consumer acceptability.

Another impact of hydrolysis upon sensory quality of whey protein is the effect of peptide buffering on acidic products, particularly beverages. Hydrolysis of proteins results

in increased exposure of carboxyl and amine groups and a subsequent increase in buffering capacity of the protein solution. This necessitates a greater amount of acid to lower the pH to desirable levels, and in the process contributes to extreme sourness and astringency.

Protein hydrolysates also face challenges with shelf-life and deteriorative reactions. All chemical reactions, such as loss of free amino groups due to non-enzymatic browning, and protein-protein aggregation, among others, proceed more quickly with protein hydrolysates due to increased molecular mobility as well as increased exposure of reactive groups, such as thiol and amino groups as well as hydrophobic residues (Netto et al. 1998; Zhou and Labuza 2007). These chemical reactions often lead to challenges such as browning, hardening or caking (Rao, Fisher, et al. 2013), and overall reduced sensory acceptability. Protein hydrolysates have also been observed to decrease the glass transition temperature of protein powder systems, making the system less stable and resulting in even greater molecular mobility (Netto et al. 1998; Zhou and Labuza 2007; Rao and Labuza 2012).

A wealth of research is available on the many beneficial hydrolysates that can be generated from whey protein including those with bioactive peptides, decreased allergenicity, improved digestibility, and enhanced functional properties. However, it is vital that the challenges facing hydrolysate usage are further investigated including improving the sensory quality and shelf life stability of WPH and the products they are added to. This is essential if WPH is to have greater industrial production and utilization in food products.

1.7 Moisture-Induced Protein/Peptide Aggregation

Though concentrated whey protein powders have shown immense promise in a variety of applications due to their high nutritional and functional qualities, there are still limitations to their use in products due to problems with aggregation during extended storage. Aggregation of whey and other proteins can occur both during storage of the isolate or hydrolysate powders or later after inclusion in high-protein food systems such as

bars (Netto et al. 1998; Zhou et al. 2008; McMahon et al. 2009; Zhu and Labuza 2010; Rao et al. 2013; Zhou et al. 2013).

1.7.1 Mechanisms of Protein/Peptide Aggregation

Protein aggregation refers to the clustering of protein molecules and the formation of aggregates through both covalent and non-covalent interactions, which are generally irreversible once they are formed (Weiss et al. 2009). Aggregate formation often leads to reduced solubility, and increased turbidity, precipitation, and formation of undesired gel networks, depending on a variety of factors such as protein concentration, presence of salts and other compounds, relative humidity, and the type and structure of aggregates formed (Zhou et al. 2008a; Zhu & Labuza 2010). Additionally, problems with solubility and precipitation can cause reduced overall functionality (De Wit 1990) and nutritional bioavailability, and thus are serious concerns limiting the usage of these ingredients.

The two primary methods by which proteins form aggregates are hydrophobic interactions and intermolecular disulfide-bond formation et (Costantino et al. 1994). The former involves the clustering of exposed hydrophobic regions of multiple protein molecules in order to decrease their unfavorable interactions with water. The latter involves the formation of new intermolecular disulfide bonds from reactive free thiol groups of protein molecules, or free thiol-disulfide interchange between protein molecules, where an intramolecular disulfide bond is replaced by an intermolecular disulfide bond. Researchers have suggested that disulfide bond formation is the more responsible of the two for whey protein aggregation (Costantino et al. 1994; Visschers & de Jongh 2005; Zhou et al. 2008a).

Researchers have also found that aggregation is often preceded by denaturation (De Wit 1990; Ju and Kilara 1998; Havea et al. 2002), and that a greater degree of denaturation often leads to a greater extent of aggregation (H. Singh & Havea 2003). Denaturation alters the structure of proteins and allows for greater covalent and noncovalent intermolecular interactions (H. Singh & Havea 2003). The rate of disulfide bond formation can be increased by most methods known to also induce denaturation such as heating (De Wit

1990), high pressure processing (Funtenberger et al. 1997; Patel et al. 2005; X. Liu et al. 2005), mechanical shear (Visschers & de Jongh 2005), alkaline pH (Alting et al. 2000; Mleko & Foegeding 2000) and high ionic strength (Bryant & McClements 2000; Unterhaslberger et al. 2006).

Additionally, common chemical reactions such as the Maillard reaction and lipid oxidation may cause protein polymerization and exacerbate protein aggregation. The progression of each of these reactions is complex, and the number of potential intermediate and end products is vast and highly dependent on the food system. The advanced stage of the Maillard reaction is characterized in part by the formation of high molecular weight colored compounds called melanoidins, which contribute to noticeable protein aggregation in protein systems (Zamora & Hidalgo 2005; Zhou et al. 2013). Products of lipid oxidation can result in free-radical attack on proteins in food systems leading to protein-protein cross-links (Gardner 1979; Kikugawa & Beppu 1987). Additionally, secondary products of lipid oxidation may participate in the Maillard reaction leading to more formation of high molecular weight aggregates (Zamora & Hidalgo 2005).

1.7.2 Protein/Peptide Aggregation during Storage

Much of the work on protein aggregation during storage has examined the interaction between time, temperature, and moisture. Most research suggested that as time and temperature of storage increase, so does rate of aggregation (Liu et al. 1990; Zhou et al. 2008a). The influence of temperature is critical as most chemical reaction rates increase with increase in temperature due to increase in molecular mobility (Levine & Slade 1986). Increase in temperature can also lead to denaturation (Zhou et al. 2013), which increases the aggregation rate, and the transition of proteins from their glassy to their more reactive rubbery state (Levine & Slade 1986; W.R. Liu et al. 1990). The increase in flexibility and mobility of whey proteins due to increase in storage temperature led to observable increases in the accessibility of thiol groups, which enhanced disulfide interchange (Zhou & Labuza 2007; Zhou et al. 2008b). Also researchers studying high-protein bar model systems formulated with whey protein observed greater formation of aggregates, increased

hardness, and changes in protein microstructure with higher storage temperature (Zhou et al. 2008a). Therefore, research suggests that storage should ideally be done at low temperatures, where formation of aggregates is generally decreased.

Moisture has similarly important effects on reaction rates and aggregation. At water activities of the monolayer, chemical reaction rates are at a minimum, since water is often a vital participant in chemical reactions (Labuza et al. 1970). Water also lowers the glass transition temperature of proteins, allowing them to more easily transition from their glassy to rubbery states and contributes to greater inter-chain mobility and greater reaction rate by serving as a reactant or reaction medium (Slade et al. 1991). Increases in moisture content of whey protein powders has led to lower denaturation temperatures, while aggregation rates decreased as moisture content dropped below 30% for whey protein powders (Zhou & Labuza 2007). Increasing moisture content resulted in increased aggregation rates until the point at which additional water caused dilution of the protein reactants, around 70-80% for a whey protein-buffer system (Taoukis et al. 1988; Zhou and Labuza 2007).

1.7.3 Proposed Solutions to the Problem of Protein/Peptide Aggregation

The challenge of combating protein/peptide aggregation in protein products has been difficult, as it can be initiated and enhanced by a variety of factors. Several potential solutions have been proposed including environmental controls, use of additives, and protein modification.

As the aggregation rate is highest at high temperature and humidity (Costantino et al. 1994), controlling storage conditions is the most widely used approach for limiting the extent of protein aggregation during storage. Most protein powders are dried to low moisture levels well below the monolayer moisture value and are then packaged in materials such as foil-lined or metalized plastics that help control the microenvironment of the powder by limiting water vapor transmission into the container. Most manufacturers will also suggest the product be stored in cool, dry areas to limit aggregation.

A number of additives have also been used with success to decrease the aggregation of protein products. Addition of sodium chloride at 0.05-0.45M levels were observed to decrease aggregation of whey protein in a model buffer system (Zhou et al. 2008a). This is believed to be due to the ability of moderate ionic strength to enhance ionic intermolecular repulsions. Thiol-blockers such as N-ethylmaledimide, 2-indoacetamide, or iodoacetic acid have been observed to decrease thiol-disulfide interchange, and reducing agents such as β-mercaptoethanol have been used to break disulfide bonds formed during aggregation, but all of these compounds are toxic and so cannot be used in foods (Zhu & Labuza 2010). In contrast, glutathione and cysteine, which are reducing agents and GRAS food ingredients, have prevented the formation of disulfide-bonded aggregates in whey protein isolate-based high protein bar model systems (Funtenberger et al. 1997; Zhu and Labuza 2010). Additionally, cupric ions can be used to reduce aggregation of proteins by oxidizing thiols and making them less reactive, and thus less likely to participate in intermolecular disulfide interchange (Hopton et al. 1989; Costantino et al. 1994).

As certain food product applications may limit the use of particular additives, researchers have also investigated protein modification techniques that may result in decreasing protein/peptide aggregation. Whey protein hydrolysates are currently used in the protein bar industry to reduce bar hardening because they serve as a plasticizer and decrease the glass transition temperature of the bar matrix (McMahon et al. 2009; Rao, Kamdar, et al. 2013). However the use of protein hydrolysates comes with its own challenges in terms of shelf-life stability. Protein hydrolysates have been observed to have increased sensitivity to environmental conditions such as temperature and humidity due to the increased hygroscopicity and increased molecular mobility of low MW hydrolysates (Rao & Labuza 2012). Hydrolysates also display increased reactivity in the Maillard reaction leading to the formation of high-molecular weight aggregates as reaction products due to increased accessibility of reactive groups such as cysteines, lysines, and n-terminal free amines (Rao & Labuza 2012). Additionally, protein hydrolysates decrease the stability of the protein system as a whole by reduction of the glass transition temperature, leading to greater molecular mobility and instability (Rao & Labuza 2012).

An ideal solution to the challenges faced with the use of protein hydrolysates would be one that curbs the stability challenges, while maintaining or enhancing the nutritional quality, functionality, and other beneficial attributes of protein hydrolysates. Recently, protein glycation via the initial stage of the Maillard reaction has been shown to decrease aggregation naturally via a number of mechanisms (Jimenez-Castano et al. 2005; Wang & Ismail 2012).

1.8 Maillard-Induced Protein Glycation

The Maillard reaction is a fundamental food chemistry reaction known best for being responsible for the development of the aromas and brown colors associated with the heat treatment of food. The Maillard reaction is actually a complex series of reactions that generate a wide variety of products, and is also often referred to as non-enzymatic browning (Hodge 1953).

1.8.1 Significance and Mechanisms of the Maillard Reaction in Foods

The Maillard reaction is often described in terms of three stages: initial, intermediate, and final; of which the first is best understood. The first description of the initial stage of the reaction is commonly attributed to Louis Camille Maillard, a French chemist in 1912 after whom the reaction was later named. The first step involves the condensation of the free aldehyde of a carbonyl containing compound with an amino group. In foods the carbonyl-containing compound is often a carbohydrate, though ascorbic acid, products of polyphenol oxidase action, and low molecular weight aroma compounds can also participate in the reaction (Labuza & Schmidl 1986). The amine groups are typically supplied by protein. This initial step results in the formation of a Schiff base, which rearranges in a step known as Amadori rearrangement, to form a ketosamine often called the Amadori compound (Hodge 1953). The Amadori compound is the first stable intermediate of the reaction (Hodge 1953).

The intermediate and final stages of the Maillard reaction involve the degradation of the Amadori compound to yield a variety of advanced Maillard reaction products (Hodge 1953). The mechanisms of these later stages of the Maillard reaction are more divergent, and may involve a variety of different steps including fissions, cyclizations, and polymerizations leading to diverse products like low molecular weight aromatic compounds, and highly polymerized and dark-colored melanoidins (Hodge 1953).

1.8.2 Control of the Maillard Reaction in Foods

In some food products such as breads and meats the formation of aromas and brown color by the Maillard reaction are desirable; however, in many other foods containing protein and reducing carbohydrates, it is important to minimize the reaction to prevent losses in quality. The rate and extent of the Maillard reaction in food products can be controlled through a variety of avenues.

The Maillard reaction proceeds the fastest at elevated temperatures and the reaction is zero-order with respect to temperature when reactants aren't limiting such as in many food products (Stamp & Labuza 1983). In many cases such as in dried milk protein powders, the reaction does still occur spontaneously at room temperatures, albeit more slowly. The reaction is also highest in systems with a water activity of 0.5-0.8. This is because available water causes increases in the molecular mobility of the reactants, and this mobility is much lower at water activities below 0.5 particularly at the monolayer value (Eichner and Karel 1971; Duckworth 1981). The reaction peaks around a water activity of 0.6 and begins to decrease as increases in available water result in dilution of the reactants (Eichner & Karel 1971). At basic pH, the rate of the Maillard reaction is the greatest. At pH above their pKa, amine groups are not protonated and so are more reactive (Labuza & Schmidl 1986). However, the rate of the reaction can also be high at extremely acidic pH (<3) as this may cause protein hydrolysis, resulting in an increase of free amine groups available for the reaction (Labuza & Schmidl 1986). To control the rate of Maillard reaction, food products are typically kept under controlled storage where the effects of temperature, water activity, and pH can be controlled.

The reaction can also be limited through the removal of reducing sugars, but as that may not be possible in all product formulations, selection of appropriate reactants is often used instead. Smaller pentose sugars proceed through the reaction much quicker than hexoses, disaccharides, or polysaccharides (Prabhakaram and Ortwerth 1994; Nacka et al. 1998; Aoki et al. 2001; Oliver et al. 2006; Laroque et al. 2008; Hiller and Lorenzen 2010; Haar et al. 2011). Larger sugars proceed more slowly in the reaction due to steric hindrance and decreased accessibility of the free aldehyde necessary for the reaction (Haar et al. 2011). Also, in the case of oligo- and polysaccharides, the shorter the carbohydrate chain, the more open chain form is present at any given time; the open chain form of a carbohydrate is the most reactive form in the reaction (Chevalier, Chobert, Dalgalarrondo, et al. 2001). Additionally, the use of charged sugars can decrease the progress of the reaction by instigating electrostatic repulsions between the proteins and sugars (Haar et al. 2011).

The protein source can also be used to control the rate and extent of the Maillard reaction in food products. Protein sources with a greater number of accessible free amine groups, such as protein hydrolysates, have a greater potential for participation in the reaction. The amino acid lysine, which contains an ε-amine group in its side chain, is considered to be the most important source of reactive amine groups in the Maillard reaction (Labuza and Schmidl 1986; Schnickels et al. 1976). Therefore, proteins with a high percentage of lysine in their primary structure, such as whey protein, have a greater potential for participation in the reaction. The ratio of amine groups to free carbonyl groups can also control the rate and extent of the reaction (Kearsley 1978); an increased ratio of carbohydrate to amino group yields the highest rate of the Maillard reaction in foods (Lea & Hannan 1951; Wolfrom et al. 1974; Warmbier et al. 1976).

Other components present in foods can also have an effect on the rate and extent of the Maillard reaction in foods. Presence of certain metals such as iron and copper can increase the rate of the reaction by aiding sugars in the redox reaction required to convert from their ring form to the more reactive open-chain form (Labuza & Schmidl 1986). Also,

presence of sulfites can decrease the rate of the reaction by keeping carbonyls in less stable conformations (Burton et al. 1963).

Extensive research has been carried out on the natural propagation of the Maillard reaction in food systems, and how best to characterize and control its proliferation to maintain food quality. However, a new area of research focused on the Maillard glycation aims at using the reaction to naturally enhance the properties of food proteins.

1.8.3 Production of Ingredients with Novel Functionality and Application Using the Maillard Reaction

While the initial research on the Maillard reaction was primarily directed on understanding the mechanisms and control of the reaction, in the past few decades new interest is evolving around the use of the Maillard reaction to impact the functionality and application of food proteins. The Maillard-induced glycation resulted in improved solubility (Chevalier et al. 2001; Jimenez-Castano et al. 2005; Wang and Ismail 2012), gelation (Li et al. 2005; Spotti et al. 2013), foaming (Chevalier et al. 2001; Mishra et al. 2001; Hiller & Lorenzen 2010), emulsification (Chevalier et al. 2001; Akhtar & Dickinson 2003, 2007; Hiller & Lorenzen 2010), and thermal stability (Hattori et al. 1997; Chevalier et al. 2001; Jimenez-Castano et al. 2005; Hiller & Lorenzen 2010; Wang & Ismail 2012) of whey protein. Researchers have also observed that Maillard-induced glycation can improve other properties of food proteins such as digestibility and antioxidant capacity of food proteins (Calligaris et al. 2004; Hiller & Lorenzen 2010; Wang & Ismail 2012).

Several researchers have studied the impact of various reaction conditions on the functionality of the end product. Researchers found that glycation of whey protein through dry heating with 15-25 kDa dextran improved mechanical properties of heat-induced gels, and that the time of the dry heating affected the gel properties (Spotti et al. 2013). Jimenez-Castano and others ((2005) examined the glycation of β-lactoglobulin with dextran and found that the best conditions for optimum production of the Amadori product was dry heating at 60°C with 44% relative humidity, and a ratio of dextran to β-lactoglobulin of 2:1. They also found that glycation with 10kDa dextran improved the solubility of β-lactoglobulin at low pH, and in other studies, improved the thermal stability of individual

whey proteins (Jiménez-Castaño et al. 2005, 2007). Chevalier and others (2001a; 2001b) found that glycation of β-lactoglobulin with small sugars resulted in an acidic shift of the isoelectric point towards a lower pH than native β-lactoglobulin, and improvements in the protein's solubility and thermal stability at acidic pH. Glycation of whey protein isolate with 500kDa dextran and also with maltodextran, resulted in a product with improved emulsification properties (Akhtar & Dickinson 2003, 2007).

Maillard-induced glycation has been shown to decrease protein/peptide aggregation in certain food systems. Researchers have observed that Maillard-induced glycation decreases protein aggregation during heating around the protein's isoelectric point, leading to maintained solubility at fairly low protein concentrations (Chevalier et al. 2001). Wang and Ismail (2012) observed that controlled and limitation glycation of whey protein with 10kDa dextran, after removal of unreacted dextran, resulted in a product that maximized protein retention and resisted aggregation and precipitation even at pH around the isoelectric point of whey protein, at protein concentrations in excess of 4%, and after heating at 80°C for 30 min. The researchers attributed this observation to structural changes including a shift to a more acidic isoelectric point, reduced surface hydrophobicity and surface sulfhydryl group exposure, an increase in net negative charge due to blockage of charged lysine residues, an increase in structural rigidity due to ionization of carbonyl groups and rearrangement of disulfide linkages, and intermolecular steric hindrance due to the attachment of the bulky sugars (Wang & Ismail 2012; Wang et al. 2013). Additionally, partial glycation was observed to improve thermal stability, causing a significant increase in the denaturation temperature of both β-lactoglobulin and α-lactalbumin, decrease in heat denaturation rate, and minimal changes to surface hydrophobicity and surface sulfhydryl group exposure even with extended heating at 80°C (Wang et al. 2013).

Despite the flurry of research on the functional benefits of Maillard-induced glycation of food proteins there are still important areas of investigation that are severely lacking. Many researchers have identified mechanisms of improved solubility and decreased aggregation of glycated protein in solution, but no work has yet investigated any potential benefit to protein powders or intermediate-moisture-foods, where moisture-

induced aggregation is a serious problem. Additionally, though there is exhaustive literature available on the glycation of any number of available protein isolates and concentrates using a wide variety of carbonyl sources, no work has yet been approached to use Maillard-induced glycation to address the challenges of protein hydrolysate stability.

Lastly, much of the work on protein glycation for functional benefit has been exploratory in nature, and doesn't reflect the needs of industrial production and application such as maximizing input usage, minimizing waste streams, product consistency, and using industry-friendly materials and techniques. For protein glycation technologies to advance pass the bench-top stage and realize their proclaimed benefit, it is vital that research also be carried out with industry friendly practices.

1.8.4 Consequences of the Maillard Reaction

Despite the promise of the Maillard reaction to improve the functionality and stability of food proteins, it does come with some drawbacks. Various intermediates and products of the Maillard reaction such as heterocyclic amines are suspected or confirmed carcinogens with high mutagenicity (Finot and Magnenat 1981; Finot 1990; Brands et al. 2000). Additionally, the Maillard reaction may cause decreases in protein quality via decreases in digestibility (Erbersdobler et al. 1981), and in losses of the essential amino acid lysine (Warren & Labuza 1977). This is all in addition to the polymerization stimulated in the later stages of the Maillard reaction and production of certain Maillard reaction products that can stimulate protein/peptide aggregation, potentially further decreasing protein quality, digestibility, and sensory acceptability. Therefore, when producing glycated protein ingredients it is of utmost importance to monitor and control the progression of the Maillard reaction in foods to the initial stages by carefully selecting the ideal substrates, reactants, and environmental conditions in order to meet the requirements of a selected application.

1.9 Conclusions

Whey protein is a highly nutritious protein source with excellent functionality, making it ideal for many food applications. Whey protein hydrolysates, in particular, have been used in many products for their enhanced functionality and health properties compared to their native counterparts. However, WPH have stability changes, mainly attributed to increased rates of deteriorative chemical reactions, such as moisture-induced protein/peptide aggregation and the Maillard reaction. These challenges are further exacerbated if WPH is exposed to non-ideal storage conditions, including high temperature and high humidity, potentially leading to loss of functionality and nutritional quality. Maillard-induced glycation has been shown to reduce protein/peptide aggregation of whey proteins in solution, and shows potential for reducing moisture-induced aggregation of WPH powder during storage. No work has yet been done on the effects of Maillard-induced glycation on reduction of moisture-induced aggregation of protein hydrolysate powders during storage. Furthermore, research must be designed to simulate industry-friendly approaches where possible to help approximate the use of these technologies in the real world.

2. Production and Characterization of a Partially-Glycated Whey Protein Hydrolysate

2.1 Overview

Partial Maillard-induced glycation of WPH was investigated over 12-120 h of incubation at 60°C, 49% relative humidity (RH), and a 4:1 ratio of dextran to protein. Glycation was initiated within 12 h of incubation, confirmed by the detection of Amadori compound formation at 304 nm. Minimal browning was detected over the entire 120 h of incubation, however, fluorescent compounds, indicators of progression to the intermediate and final stages of the Maillard reaction, were detected as early as 12 h, and peaked at 120 h of incubation. Detection of Amadori compound formation and loss of free amino groups indicated that the progression of the reaction increased with time as expected. PGWPH produced upon incubation for 48 h was selected for investigation in further studies, due to its moderate level of Amadori compound formation, with minimal progression to intermediate and final stages of the Maillard reaction. Free dextran was removed from glycated and non-glycated protein and peptides with moderate success using a 2-step membrane filtration and hydrophobic interaction chromatography (HIC). The final composition of glycated whey protein hydrolysate was approximately 1:1 protein to carbohydrate. Purified glycated whey protein hydrolysate displayed minimal blockage of the essential amino acid lysine (4.4%), and no significant decrease in digestibility compared to WPH. Overall, this work showed that Maillard glycation can be induced and controlled to low-levels in whey protein hydrolysate, producing a value-added product with maintained nutritional quality, and potentially enhanced functionality and stability.

2.2 Introduction

Whey protein is a commonly consumed protein source, popular due to its high nutritional quality and excellent functionality. Whey protein is highly digestible and contains all essential amino acids in the relative proportions required for health (Walstra et al. 2006). It is also an excellent source of the branched chain amino acids isoleucine, leucine and lysine, valued by consumers for their contributions to optimal muscle synthesis and repair (Page et al. 2004). Whey protein displays excellent functionality including gelation (Singh & Havea 2003), emulsification (Kilara 1994), foaming (Mangino et al. 1987) and high solubility across a broad range of pH (Mulvihill & Ennis 2003). These properties have led to the inclusion of whey protein into a diverse array of products from baked goods to dairy products to consumer protein supplementation products, leading to an estimated global market value of 9.8 billion U.S.D. (in 2013) (Affertsholt 2009).

The nutritional and functional quality of whey protein along with its high market value make whey protein an ideal substrate for production of value-added ingredients such as whey protein hydrolysate. Whey protein hydrolysates have several benefits over traditional isolates including the presence of bioactive peptides (FitzGerald & Meisel 2003), increased digestibility (Sinha et al. 2007), and improved functionality (Mullally et al. 1994; Foegeding et al. 2002; Creusot & Gruppen 2007), and thermal stability (Foegeding et al. 2002). However, a challenge with the commercial use of hydrolysates in food products is their increased reactivity in many deteriorative reactions including moisture-induced protein/peptide aggregation leading to decreased shelf-life and sensory quality (Netto et al. 1998; Zhou & Labuza 2007). Hydrolysates are not yet as widely used as protein isolates, but their growth is greatly outpacing that of traditional protein isolates (Affertsholt 2010) and so it is important that their stability challenges be addressed.

Maillard-induced glycation is a protein modification approach that has recently been researched for its potential to create protein ingredients with enhanced functionality and stability. The initial stage of the Maillard reaction is a condensation reaction between protein/peptides and reducing sugars leading to the formation of glyco-conjugated proteins that often display new beneficial functional attributes. However, progression of the

Maillard reaction beyond its initial stages can lead to the formation of carcinogenic byproducts (Brands et al. 2000), decreased digestibility (Erbersdobler et al. 1981), and losses of the essential amino acid lysine (Warren & Labuza 1977). Therefore, the reaction is often induced and controlled to limit propagation to advanced stages (Wang & Ismail 2012). The reaction is controlled by regulating important parameters including time, temperature, relative humidity (RH), carbohydrate type, and ratio of protein to carbohydrate.

Maillard-induced glycation of whey protein has been shown to improve its solubility (Chevalier 2001; Jimenez-Castano et al. 2006; Wang and Ismail 2012), gelation properties (Li et al. 2005; Spotti et al. 2013), foaming ability (Chevalier et al. 2001; Mishra et al. 2001; Hiller and Lorenzen 2010), emulsification capacity and stability (Chevalier et al. 2001; Akhtar and Dickinson 2002), as well as thermal stability (Chevalier 2001; Jimenez-Castano et al. 2006; Wang and Ismail 2012). It is, therefore, hypothesized that limited and controlled Maillard-induced glycation of whey protein hydrolysate will generate a product with maintained nutritional quality and enhanced functionality. Thus, it is our objective to produce and characterize a partially-glycated whey protein hydrolysate product using controlled and limited Maillard-induced glycation, assimilating industrial procedures where possible.

2.3 Materials and Methods

2.3.1 Materials

Whey protein isolate (WPI) (BiPRO®, 92% protein) and whey protein hydrolysate (WPH) (BioZate®, 90% protein) were generously provided by Davisco Foods International, Inc. (Eden Prairie, MN, USA). Dextran, average molecular weight of 9-11 kDa (D9260) (dextrose equivalence: 2.3), was purchased from Sigma-Aldrich (St. Louis, MO, USA). Bovine serum albumin liquid standard (23210), Pierce™ bicinchonic acid protein assay kit (23227), and Pierce™ glycoprotein staining kit (24562) were purchased from Thermo Scientific (Waltham, MA, USA). Tris-tricine Criterion™ precast peptide gels (345-0068), tricine sample buffer (161-0739), Precision Plus Protein™ prestained molecular weight ladder (161-D377), and concentrated tris-tricine-sodium dodecyl sulfate running buffer (161-0744) were purchased from Bio-Rad (Hercules, CA, USA). Opaque, black (Costar® 3916); opaque, white (Falcon® 35-3296); and UV-specific (Costar® 3635) 96-well polystyrene microplates were purchased from Corning, Inc. (Corning, NY, USA). Pronase enzyme from *Streptomyces griseus* was purchased from Roche Diagnostics (Indianapolis, IN, USA). Phenyl sepharose High Performance media (17-1082-01) and phenyl sepharose CL-4B media (17-0810-01) were purchased from GE Healthcare (Little Chalfont, UK). Amicon® 3kDa centrifugal filtration devices (UFC900324) and Pellicon® 3 membranes (P3C005C01) were purchased from EMD Millipore (Billerica, MA, USA). Celltreat® 0.45um filters were purchased from Celltreat Scientific Products (Shirley, MA, USA). Sep-Pak C18 filter cartridges were purchased from the Waters Corporation (Milford, MA, USA). Furosine standards (SC494) were purchased from the PolyPeptide Group (San Diego, CA, USA). Pepsin enzyme (EC 3.4.23.1, P6887) and trypsin enzyme (EC 3.4.21.4, T8003) were purchased from Sigma-Aldrich. All other reagent grade chemicals were purchased from Fisher Scientific (Waltham, MA, USA) or Sigma-Aldrich.

2.3.2 Controlled Maillard Glycation of Whey Protein Hydrolysate

WPH and dextran were mixed in a 1:4 (w/w) ratio, dissolved in 0.01 M phosphate buffer, pH 7, and lyophilized. The dried mixture was then pulverized to a powder

consistency, and incubated in triplicate in sealed desiccators under conditions of 49% relative humidity (RH) and 60°C. WPH that had previously been dissolved in 0.01 M phosphate buffer, but without the addition of dextran served as a control. RH of the desiccators was maintained using saturated sodium bromide slurries (49% RH at 60°C). Samples were removed from the desiccators at predetermined time-points from 12-120 hours to examine the effects of time on the extent of glycatin, and all samples were then stored at -20°C until analysis.

2.3.3 Estimation of Maillard Glycation Extent of WPH Incubated with Dextran

To estimate the extent of glycation that occurred over the course of the 12-120 hours of incubation, and to aid in selecting an optimum incubation time for production of glycated WPH for further study, a variety of analyses including estimation of Amadori compounds and browning, estimation of fluorescent Maillard compounds, estimation of amino group loss, and visualization of protein/peptide molecular weight distribution following sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were carried out.

2.3.3.1 Estimation of Amadori Compound Formation and Browning

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

2.3.3.2 Determination of Fluorescent Compounds

The change in fluorescent compound formation in WPH incubated with dextran over time was monitored as described by Rao et al. (2012), with modifications. WPH incubated with dextran, WPH incubated without dextran, and non-incubated controls were analyzed in triplicate. For each, a 1.0 % protein solution (w/v) was prepared in DDW. A 0.178 mL aliquot of pronase solution (20 U/mL pronase in 50 mM tris-HCl, pH 7.2), made fresh daily, was added to each sample, which was then incubated on a carousel mixer for 50 min at room temperature to ensure complete digestion. After digestion, the samples were centrifuged at 15,682 x g for 15 min, and the supernatant was removed and diluted 1:1 (v/v) with phosphate buffered saline solution (20 mM phosphate buffer, 15 mM NaCl, pH 7.0). A 200 µL aliquot of each sample was placed into the wells of a black, opaque microplate, and the fluorescence intensity (FI) was measured at an excitation of 360 nm (bandwidth 40 nm) and emission of 460 nm (bandwidth 30 nm). Readings were corrected for protein content using the Thermo Scientific Pierce™ bicinchonic acid (BCA) assay kit, following the manufacturer's instructions. The fluorescence intensity (FI) of samples was determined by first correcting for the protein content of the sample. Then the FI values of all incubated samples were adjusted by subtracting the FI values of their respective non-incubated controls, which served as blanks. The FI values were then corrected to the FI of 5 ppm quinine sulfate solution which was prepared diluting quinine sulfate sulfuric acid solution (100 µg/mL quinine sulfate in 50 mM sulfuric acid), prepared fresh daily, to 5 ppm with phosphate buffered saline solution, as in **Equation 1**.

Equation 1:

$$Fluorescence\ Intensity\ (FI)/g\ protein = \left(\frac{\frac{Emi_{Sample}}{g\ Protein} - \frac{Emi_{Control}}{g\ Protein}}{Emi_{5\ ppm\ Quinine\ Sulfate}} \right)$$

Where:
Emi = emission reading at 460 nm (bandwidth 30 nm)

2.3.3.3 Loss of Free Amino Groups

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

Equation 2:

$$\%\ Loss\ in\ Free\ Amino\ Group\ Content = \frac{\%\ Free\ Amino\ Groups\ of\ Control - \%\ Free\ Amino\ Groups\ of\ Sample}{\%\ Free\ Amino\ Groups\ of\ Control} \times 100$$

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

The change in molecular size of proteins/peptides in WPH incubated with dextran over time was monitored with SDS-PAGE using tris-tricine peptide gels as outlined by

Laemmli (1970), Schägger and von Jagow (1987), with modifications. WPH incubated with dextran and non-incubated controls were analyzed in triplicate. For each, a 0.02% protein solution (w/v) was prepared in DDW before being diluted 1:1 (v/v) with tricine sample buffer containing SDS and 2% β-mercaptoethanol. All samples were then heated in boiling water for 5 min and then allowed to cool to room temperature. A 10 μL aliquot of each sample was loaded onto a precast 10-20% gradient tris-tricine peptide gel, along with a 10 μL aliquot of a prestained molecular weight ladder (Bio-rad, Hercules, CA, USA). All gels were electrophoresed with tris-tricine-SDS buffer at 4°C and 125 V for 3-4 hours. Gels were then stained using either Coomassie blue stain or the Pierce™ Glycoprotein Staining kit.

2.3.3.4.1 Coomassie Blue Staining of Polyacrylamide Gels

Coomassie blue staining was carried out by first immersing gels in Coomassie blue stain solution (45% methanol (v/v), 10% glacial acetic acid (v/v), and 0.3% Brillant Blue R250 (w/v)) for 1 hour while shaking. Destaining of the gel was then carried out by repeated rinsing of the gel with a destaining solution (10% glacial acetic acid (v/v), 5% methanol (v/v)). Visualization of the gel was done using a Canon T3i camera with the gel positioned over a fluorescent back-lit platform for optimal resolution.

2.3.3.4.2 Glycoprotein Staining of Polyacrylamide Gels

Glycoprotein staining was carried out on a second set of gels with a Pierce™ Glycoprotein Staining Kit. Staining and destaining was performed according to the manufacturer's instructions. Visualization of gels was done as outlined in **Section 2.3.3.4.1**.

2.3.4 Separation of Free Dextran from Partially Glycated Whey Protein Hydrolysate

To prevent further Maillard glycation during storage and testing of the WPH incubated with dextran, removal of dextran that did not react to form a glycosidic bond with a protein/peptide over the course of the incubation period, hereafter referred to as 'free dextran', was desired. Separation of the free dextran from the glycated and non-glycated

protein/peptides, hereafter referred as 'partially glycated whey protein hydrolysate' (PGWPH), using hydrophobic interaction chromatography (HIC) as outlined by Wang and Ismail (2012) was explored. Modifications including the use of centrifugal filtration devices and chromatographic scale-up were undertaken. All further analysis was carried out using WPH incubated with dextran for 48 hours. The selection of this sample was based on the results of estimated glycation and blockage of free amino groups, as will be discussed in the results section.

2.3.4.1 Preliminary Chromatographic Trials

Separation of the free dextran from the PGWPH was investigated using hydrophobic interaction chromatography (HIC). Initial chromatographic conditions were first tested on a non-incubated 4:1 mixture of dextran and WPH to approximate PGWPH. A Pharmacia Biotech ÄKTA fast protein liquid chromatography pump, equipped with a Shimadzu Ultra-Fast Liquid Chromatograph (UFLC) High Performance Liquid Chromatography (HPLC) CBM-20A communications bus module, SPD-20AV UV-vis detector and FRC-10A fraction collector was used. A HIC column, 20 cm x 16 mm, was packed with phenyl sepharose high performance media, and equilibrated with 1M ammonium sulfate, pH 7. A non-incubated mixture of dextran and WPH (4:1) was dissolved in DDW (4% protein, w/v), and a 2 mL aliquot of the solution was injected onto the column at a flow rate of 3 mL/min. Dextran was eluted from the column using 60 mL of 1M ammonium sulfate (0-20 min), followed by a 60 mL DDW rinse (20-40 min) to elute protein and peptides. Elution of the protein/peptides in WPH was monitored at 220 nm, using EZStart™ software (Shimadzu, Kyoto, Japan). Elution volumes were experimentally adjusted to achieve the best possible separation of dextran and recovery of protein/peptides. Dextran content of the collected fractions (28 total 3mL fractions, collected each min) were determined experimentally using the AOAC phenol-sulfuric acid method (Official Method 988.12, AOAC International, 1988), with modifications. Briefly, fractions were diluted with DDW to reach 50 ug/mL carbohydrate. A 1 mL aliquot of diluted sample was taken, and 25 μL of 80% (v/v) phenol and 2.5 mL concentrated sulfuric acid were added. All samples were vortexed for 5 s and allowed to stand for 10 min to

cool to room temperature. Glucose standards ranging from 0-100 μg/mL glucose were prepared in a similar fashion to construct a standard curve. The absorbance of each standard and diluted fraction was measured at 490 nm using a spectrophotometer.

2.3.4.2 Improvement of Free Dextran Separation Protocol with the Use of Centrifugal Filtration Devices

To improve the retention of low molecular weight peptides, the addition of a centrifugal filtration step to the separation protocol was investigated. Two test runs were conducted using WPH incubated with dextran and non-incubated WPH and dextran mixed in a 1:4 ratio. Both WPH preparations were dissolved in DDW (4.0% protein, w/v), and 7.5 mL aliquots were loaded into Amicon® 3kDa centrifugal filtration devices, which had been pre-rinsed with DDW. Solutions were centrifuged for 120 min at 5,000 x g, after which 2 mL DDW was added as a final rinse, and the solutions were centrifuged at 5,000 x g for a final 60 min. The filtration retentates and permeates were bulked and lyophilized separately. The dried retentates and permeates were solubilized in 2mL DDW, and 1 mL of that solution was separated using HIC and analyzed for carbohydrate content as outlined in **Section 2.3.4.1**. An additional test run was conducted using WPH incubated with dextran, which was prepared at a reduced 2.0% protein concentration (w/v), and 7.5 mL aliquots were loaded into centrifugal filtration devices before centrifugation and separation, as above.

To reduce the quantity of free dextran passing through the centrifugal filtration membranes and into the permeate, a final test was run on WPH incubated with dextran to investigate the efficacy of a second filtration. Dried permeate from WPH incubated with dextran filtered as above was dissolved in DDW (4.0% protein, w/v), and a 12 mL aliquot was loaded into Amicon® 3kDa centrifugal filtration devices, which had been pre-rinsed with DDW. Solutions were centrifuged for up to 60 min at 5,000 x g, and aliquots of the permeate was removed for analysis at 10 minute intervals. The carbohydrate content of the permeate fractions was analyzed as outlined in **Section 2.3.4.1**. The protein content of the permeate fractions was analyzed by Dumas using the AOAC Official Method 968.06

Dumas nitrogen combustion method (AOAC International, 1998) and a Nitrogen Analyzer (LECO® TruSpecN™, St. Joseph, MI, USA) with a conversion factor of 6.38.

2.3.4.3 Improvement of Free Dextran Separation Protocol with Chromatographic Scale-up

To improve the efficiency of the free dextran separation protocol, the chromatographic conditions were scaled-up to accommodate a higher throughput. The same chromatography system described in **Section 2.3.4.1** was used with exception of the column. A HIC column, 11 cm x 50 mm, was packed with phenyl sepharose CL-4B media, and equilibrated with 1M ammonium sulfate, pH 7. Lyophilized retentate, produced as outlined in **Section 2.3.4.2**, was dissolved in 1M ammonium sulfate (3% protein, w/v), and a 50 mL aliquot of the solution was injected onto the column at a flow rate of 10 mL/min. Free dextran was eluted from the column using 0.5 L of 1M ammonium sulfate. PGWPH was eluted with 1 L DDW. Elution of the protein/peptides in retentate PGWPH was monitored at 220 nm, as in **Section 2.3.4.1**. Elution volumes were experimentally adjusted to achieve best possible separation of free dextran and recovery of retentate PGWPH. Dextran content of the collected fractions were determined experimentally using the phenol-sulfuric acid method, as outlined in **Section 2.3.4.1**.

Eluted retentate PGWPH was collected and then desalted using a 10 kDa molecular weight cut-off Pellicon 3 membrane with 0.11 m^2 surface area (EMD Millipore, Billerica, MA, USA). The eluted retentate PGWPH was diafitered for 9 hours for 10 diavolumes until reaching a final conductivity of 165 microsiemens. The diafiltration was performed with no back pressure on the system. The lyophilized permeate, as produced in **Section 2.3.4.2**, was then dissolved in the desalted PGWPH solution, and the final PGWPH solution was lyophilized. The lyophilized PGWPH was then pulverized to a powder consistency, and stored at -20°C until needed for further testing.

2.3.5 Characterization of Partially Glycated Whey Protein Hydrolysate

After purification of PGWPH from excess free dextran, moisture, protein, and total carbohydrate contents were determined. Further characterization including % lysine

blockage and digestibility were carried out to assess changes in nutritional quality due to glycation or the purification process.

2.3.5.1 Analysis of Protein Content

Protein content of PGWPH was determined using a Nitrogen Analyzer (LECO® TruSpecN™, St. Joseph, MI, USA) following the Dumas nitrogen combustion method (Method 968.06, AOAC International, 1998). For all whey protein samples, a nitrogen conversion factor of 6.38 was used.

2.3.5.2 Analysis of Carbohydrate Content

Carbohydrate content of PGWPH was determined using the phenol-sulfuric acid method, as outlined in **Section 2.3.4.1**, with one exception. A 0.01% protein solution (w/v) of PGWPH was prepared in DDW, in duplicate, and then was analyzed.

2.3.5.3 Analysis of Moisture Content

Moisture content of PGWPH was determined following the Karl Fischer method using an Aquatest CMA Karl Fischer coulometric titrator (Photovolt Instruments, Minneapolis, MN, USA) (Fischer 1935; MacLeod 1991). A 50 mg sample of PGWPH was added to 20g methanol, in duplicate, and the samples were shaken at 100 rpm for 18 hours at room temperature in tightly sealed vials; methanol blanks were prepared in the same way, in duplicate, but were without sample. After 18 hours of extraction, 1 mL of the methanol extract of each sample or blank was removed with a syringe. A silicon stopper was used to seal the needle of the syringe to prevent evaporation of the methanol during weighing. The extract was injected into the Karl Fischer coulometric titrator to obtain 'R' (μg of water) and then the emptied syringe was weighed to verify the mass of the extract injected. Moisture content was then calculated with **Equations 3-5**.

Equation 3

$$\text{Moisture Content (mg)} = \left(\frac{\text{R}}{1000} * \frac{\text{Methanol mass (g)}}{\text{Injection mass (g)}}\right) - \text{Methanol mass (g)} * \text{Total } H_2O_{\text{blank}}$$

Equation 4

$$\%H_2O_{\mathbf{wb}} = \frac{\text{Moisture Content (mg)}}{10 * \text{Sample mass (g)}}$$

Equation 5

$$\%H_2O_{\mathbf{db}} = \frac{\%H_2O_{\mathbf{wb}} * 100}{100 - \%H_2O_{\mathbf{wb}}}$$

Where:
R = water (μg)
Methanol mass = mass of methanol sample was extracted with
Injection mass = mass of sample methanol extract injected
db = dry basis
wb = wet basis

2.3.5.4 Determination of Percent Lysine Blockage

Percent lysine blockage in PGWPH was determined by measuring 2-furoyl-methyl-Lys, furosine, which is formed upon hydrolysis of the Amadori compound obtained specifically from the interaction of lysine with a reducing carbohydrate during the Maillard reaction (Resmini et al. 1990). Furosine was quantified using the methods of Krause et al. (2003) and Jiménez-Castaño et al. (2007), with modifications. PGWPH, and non-incubated WPH, were analyzed in triplicate. For each, 3.75 mL of a 0.5% protein solution (w/v) was prepared in 7.95 N HCl in a vial, and the head-space was flushed with nitrogen to render it inert. Samples were heated at 110°C for 24 hours. After allowing samples to reach room temperature, the supernatant of each sample was removed and centrifuged at 10,000 x g for 1 min to settle any debris from the acid digestion. Sample supernatants were then filtered through Celltreat® 0.45um filters (Celltreat Scientific Products, Shirley, MA, USA). Sep-Pak C18 cartridges (Waters Corporation, Milford, MA, USA) were then pre-wet and activated first with 2 mL methanol followed by 2mL DDW before applying 0.5 mL of the samples at a rate of 0.5 mL/min. The furosine in the sample was then eluted by passing 2 mL of 3N HCl through the cartridge at a rate of 1 mL/min. The eluted furosine was neutralized with the addition of 0.33 mL 18M NaOH.

The furosine content of all samples was then quantified using HPLC based on the method outlined by Jiménez-Castaño et al. (2007). An HPLC system (Shimadzu Corp., Kyoto, Japan) equipped with a SIL-10AF auto injector, CTO-20A oven, and SPD-M20A

photo diode array detector was utilized. 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. The elution program was isocratic maintaining 15% acetonitrile containing 5mM sodium heptanesulphonate and 0.2% (v/v) formic acid throughout the run. A 20 μL aliquot of the neutralized furosine-eluted sample was injected onto the column at a flow rate of 1.2 mL/min. The column temperature was maintained at 35°C throughout the run, and UV detection was measured at 280nm. Furosine was quantified based upon its peak area at the retention time of approximately 10.5 min. Calibration and standard curve generation was carried out using furosine standards of 0.5-20 ppm.

2.3.5.5 Determination of *In-vitro* Digestibility

Digestibility of PGWPH was evaluated using sequential *in-vitro* digestion with the enzymes pepsin and trypsin as outlined by Tang et al. (2006) and Chevalier et al. (2001), with modifications. PGWPH and non-incubated WPH, were analyzed in duplicate. For each, a 1% protein solution (w/v) was prepared in 0.1 N HCl. Samples were incubated in a 37°C water bath for 10 min followed by the addition of pepsin (50 μL of 3 mg/mL). Samples were further incubated in the 37°C water bath for 2 hours before the pH was adjusted to 7.0 using 1.0 N NaOH to terminate pepsin activity, at which point trypsin was added (50 μL of 8 mg/mL). The pH of all samples was monitored and adjusted during incubation to maintain the optimum pH of 7.0 for the trypsin enzyme. After 2 hours of incubation with trypsin, the samples were filtered through 0.45 μm filters and immediately frozen at -20°C until further analysis.

The peptide profiles of the digested samples and non-digested controls were determined using HPLC analysis as outlined by Chevalier et al. (2001). The same HPLC as described in **Section 2.3.5.4** 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. The elution program was a binary gradient of HPLC-grade water (solvent A) and acetonitrile (solvent B), both containing 0.11% (v/v) trifluoroacetic acid. After injecting a 20 μL aliquot of the digested samples or controls onto the column, solvent B was linearly increased from 16% to 40% in 20 min, kept constant for 10 min, increased

to 80% for 10 min, and then decreased to 16% in 10 min. Throughout the run, the column temperature was maintained at 35°C and the flow rate was kept constant at 1.2 mL/min. The eluted peptides were monitored at 214 nm. Percent digestibility was determined based on the differences in select peak areas obtained at 214 nm between digested samples and their complementary non-digested controls **(Equation 6)**.

Equation 6:

$$\text{Digestibility (\%)} = \frac{(Peak\ area_{non-digested\ control} - Peak\ area_{\ digested\ sample})}{Peak\ area_{non-digested\ control}} * 100\%$$

2.3.6 Statistical Analysis

Analysis of variance (ANOVA) was carried out using IBM SPSS Statistics software version 22.0 for Windows (SPSS, Inc., Chicago, IL, USA). Significant differences among the respective means were determined when a factor effect or an interaction was found to be significant ($P \leq 0.05$) using the Tukey-Kramer multiple means comparison test. ANOVA tables for Chapter 2 can be found in Appendix F (Tables 7-12).

2.4 Results and Discussion

2.4.1 Estimation of Maillard Glycation Extent of WPH Incubated with Dextran

The Amadori compound is the first stable intermediate of the Maillard reaction, and so its presence is frequently used to estimate the propagation of the reaction (Zhu et al. 2008; Wang & Ismail 2012). Estimation of Amadori compound formation using absorbance at 304 nm for WPH incubated with and without dextran suggested that glycation did occur upon incubation at 49% RH and 60°C (Figure 1). The reaction appeared to have been initiated within the first 12 h of incubation, as indicated by a significant difference in 304 nm absorbance between non-incubated (0h) and 12 h incubated WPH with dextran. Absorbance at 304 nm continued to significantly increase with time indicating continued formation of the Amadori compound. The plateau in 304 nm absorbance seen between 108-120 h of incubation may suggest an equilibrium of new Amadori compound formation, and conversion of the Amadori compound to intermediate and final stage Maillard reaction products (MRPs). The 304 nm absorbance of WPH incubated with and without dextran for 72 h was higher than that observed for 84 or 96 h of incubation. As samples of WPH with and without dextran for each time point were stored in separate desiccators for ease of removal during the study, it is hypothesized that this deviation is due to the isolated environment of the desiccator used for the 72 h incubated samples, which may have experienced a slightly higher RH leading to increased Amadori compound formation during incubation. Absorbance at 304 nm was much higher for WPH samples incubated with dextran than without, as expected; however, as there was a significant increase in 304 nm absorbance of WPH incubated without dextran over time, it was concluded that some Maillard reaction occurred even without dextran. This is likely attributed to Maillard reaction of WPH with the trace amount of lactose present in WPH (up to 1%, by manufacturer's specifications). These results were similar to those observed by other researchers including Wang and Ismail (2012) and Zhu et al. (2008). Wang and Ismail (2012) observed similar 304 nm absorbances after incubation of WPI with 10 kDa dextran at 60°C and 49% RH, but at a slower rate than was observed in this study, which could be due to the increased number of accessible amino groups in WPH compared to

WPI and the increased molecular mobility and reactivity of protein hydrolysates. Zhu et al. (2008) saw much higher absorbances after incubation of WPI and dextran, but incubation was carried out in a 10% WPI, 30% dextran solution at 60°C for up to 48 h.

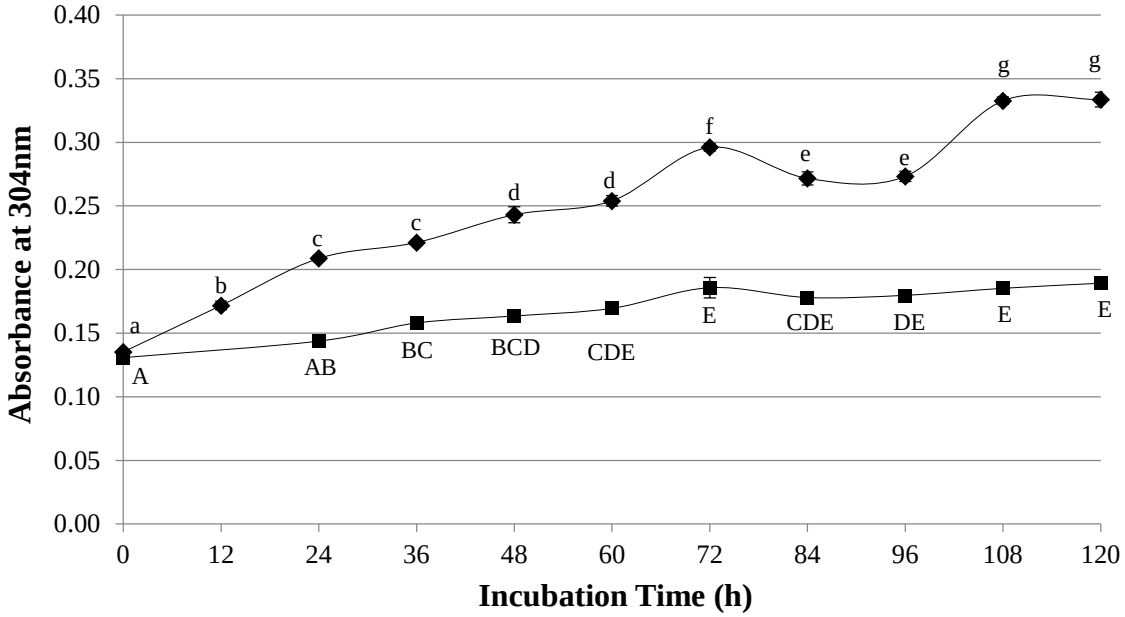

Figure 1. Amadori compound formation as determined by UV-Visible difference spectroscopy at 304 nm for whey protein hydrolysate (WPH) incubated with dextran (◆) and control WPH incubated without dextran (■) at 60°C for 0-120 h at 49% RH. Different letters above or below the shapes indicate significant differences between different time points according to the Tukey-Kramer multiple means comparison test ($P \leq 0.05$).

As the final stages of the Maillard reaction are known to give rise to MRPs such as the high molecular weight, brown-colored melanoidins, monitoring of browning, by measuring absorbance at 420 nm, is frequently carried out to estimate progression of the Maillard reaction to its final stages (Jiménez-Castaño et al. 2005; Corzo-Martinez et al. 2008; Wang & Ismail 2012). Estimation of browning using absorbance at 420 nm suggested that browning significantly increased over time for WPH incubated both with and without dextran (Figure 18 in Appendix A). However, the absorbance at 420 nm was maintained below 0.030, even after 120h of incubation, and so suggested minimal production of melanoidins. These results were similar to other researchers' findings (Jimenez-Castano et al. 2005a; Wang and Ismail 2012), though slightly higher, perhaps

again due to the increased chemical reactivity of WPH in the Maillard reaction. However, browning was much lower than observed by Zhu et al. (2008) (in solution glycation of WPI and dextran after >24 h of incubation) and Corzo-Martinez et al. (2008) (incubation of β-lactoglobulin with galactose at 50°C and 44% RH after >24 h of incubation).

Formation of fluorescent compounds occurs during the intermediate and final stages of the Maillard reaction (Adhikari 1973; Baisier & Labuza 1992), so these markers may also be used to quantify progression of the Maillard reaction beyond the initial stage. This is of particular value when the reaction has not proceeded to the stage of melanoidin production and an increase in browning is not visible (Matiacevich & Pilarbuera 2006). The % fluorescence intensity of WPH incubated with dextran increased over time, indicating an increase in the formation of fluorescent compounds (Figure 2). It also appears that low-levels of Maillard-glycation occurred in WPH incubated without dextran, as fluorescence intensity increased with time, which is in accordance with the observations of UV-Visible difference spectroscopy. The % fluorescence intensities of the 108-120 h incubated samples support the hypothesis that an increased progression to intermediate and final stage MRPs occurred during that time, as a steep and significant increase in fluorescent compound formation is visible. Again, the values observed of WPH incubated with and without dextran for 72 h are higher than that of the neighboring time-points, suggesting that a greater amount of Maillard reaction was stimulated in these samples. It is important to note that fluorescent compounds may arise from other chemical reactions apart from the Maillard reaction including lipid oxidation (Castilho et al. 1994), however, the WPH used in this study contained less than 0.5% lipid (by manufacturer's specification) and so fluorescent compound formation is believed to be due to Maillard-glycation alone.

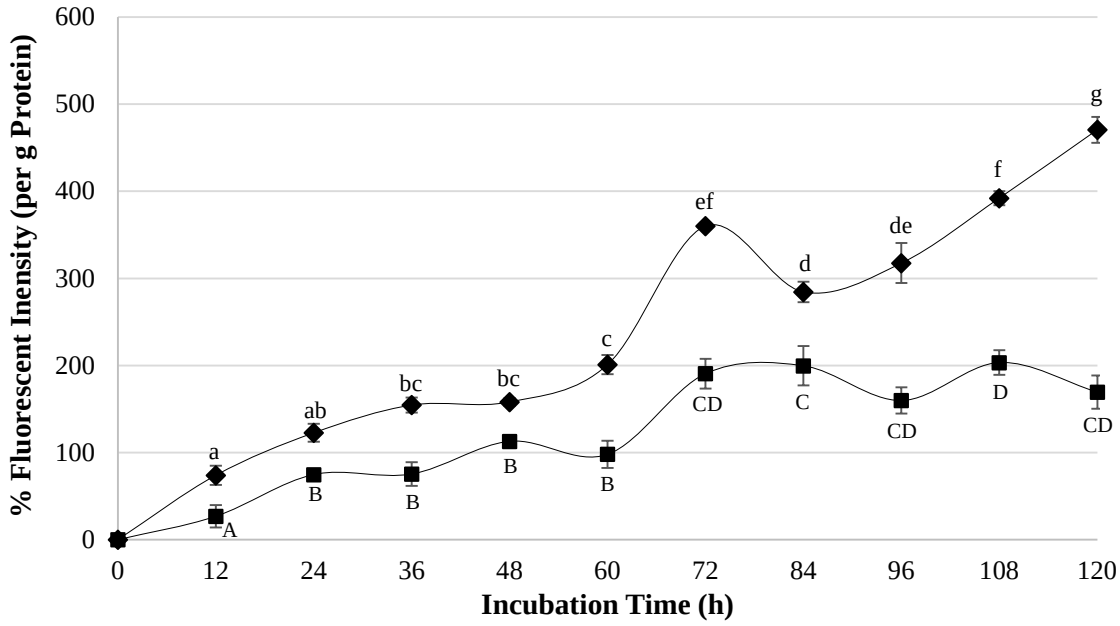

Figure 2. Fluorescent compound formation as determined by fluorescent intensity quantification (excitation: 360 nm, emission: 460 nm) for whey protein hydrolysate (WPH) incubated with dextran (◆) and control WPH incubated without dextran (■) at 60°C for 0-120 h at 49% RH. Error bars represent standard errors (n=3). Different letters above or below the shapes indicate significant differences between different time points according to the Tukey-Kramer multiple means comparison test ($P \leq 0.05$).

The amino group is one of two primary reactants of the Maillard reaction, and so its disappearance can be used to indicate the progress of the reaction (Ames 1992; Wang & Ismail 2012). Free amino group content as determined by the OPA method of WPH incubated with dextran decreased over time, as indicated by a significant increase in % loss of free amino groups (Figure 3). Extent of free amino group loss was moderate, ranging from 15.9-30.8% after 12-120 h of incubation. The % free amino groups in WPH before incubation was 11.05%, which was reduced to 8.65% after 48h of incubation, and to 7.65% after 120h of incubation with dextran at 60°C and 49% RH. A significant increase in % loss of free amino groups (15.9%) was initiated within the first 12h of incubation. This was in accordance with the observations of UV-Visible difference spectroscopy which also showed an initiation of Maillard-glycation within the first 12h of incubation, and a subsequent increase in Maillard-glycation over time. The % loss of free amino groups observed in this study was greater than that observed by other researchers, namely Wang

and Ismail (2012). The authors observed a loss of 1.4% of the available amino groups of WPI after 96 h of incubation with dextran at 60°C and 49% RH, compared to a loss of 26.6% of free amino groups after 96 has observed in this study. WPH has many more accessible amino groups, including the increased number of terminal amines and increased accessibility of lysine residues due to hydrolysis, and so along with the increased chemical reactivity of protein hydrolysates, it is not surprising that a greater reduction of free amino groups was observed.

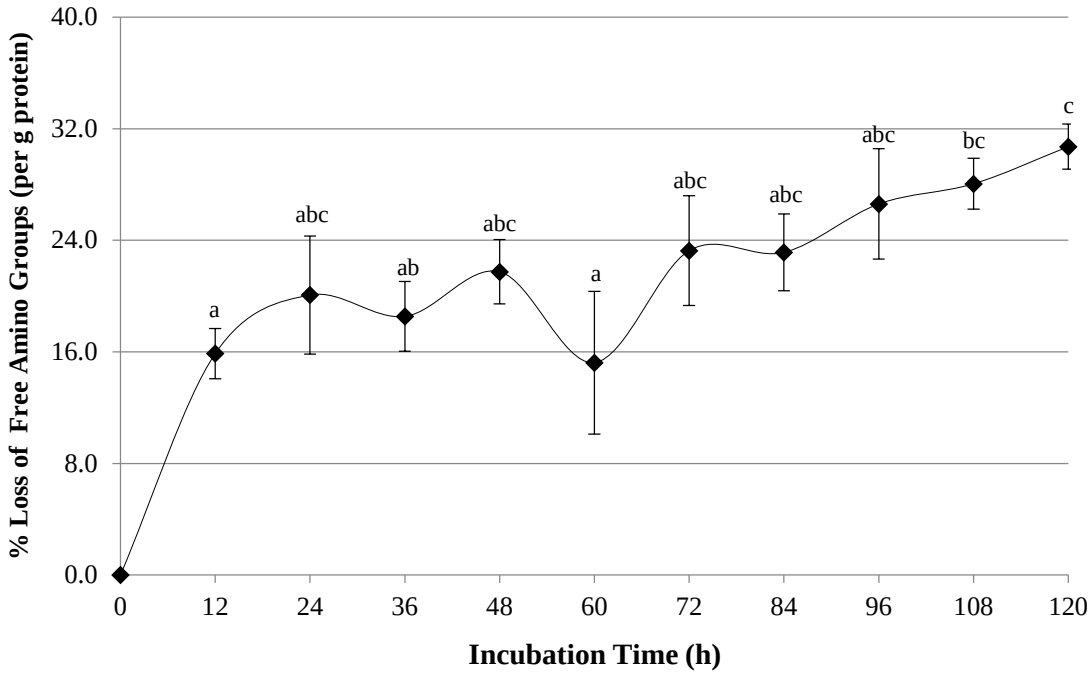

Figure 3. Percent loss of free amino groups as determined by the OPA method for whey protein hydrolysate (WPH) incubated with dextran (◆) at 60°C for 0-120 h at 49% RH. Error bars represent standard errors (n=3). Different letters above the shapes indicate significant differences between different time points according to the Tukey-Kramer multiple means comparison test ($P \leq 0.05$).

Maillard-glycation involves the covalent addition of one or more carbohydrate moieties to a protein/peptide, resulting in an increase in the molecular weight of the final glycoprotein compound (Ames 1992; Jimenez-Castano et al. 2005; Zhu et al. 2008; Wang & Ismail 2012). This increase in molecular weight of WPH proteins/peptides, due to the attachment of one or more 10 kDa dextran, can be visualized by SDS-PAGE with

Coomassie blue staining. Changes in the distribution of protein/peptide molecular weights as visualized by SDS-PAGE suggested that protein/peptide size increased over time (Figure 4). This was indicated by the apparent shift to higher molecular weights over time, leading to the observed smearing in the 15-250 kDa range, and band lightening in the 2-10 kDa range. There was an apparent heterogeneity of molecular weight increases, indicated by the longitudinal smearing over time, particularly after 96-120h of incubation. This is also in accordance with the Maillard reaction, as the number of dextran substitutions to a protein/peptide can be heterogeneous. These results are similar to those observed by other researchers including Jimenez-Castano et al. (2005b), Corzo-Martinez et al. (2008), and Wang and Ismail (2012), who all observed heterogeneous molecular weight increases after the onset of glycation.

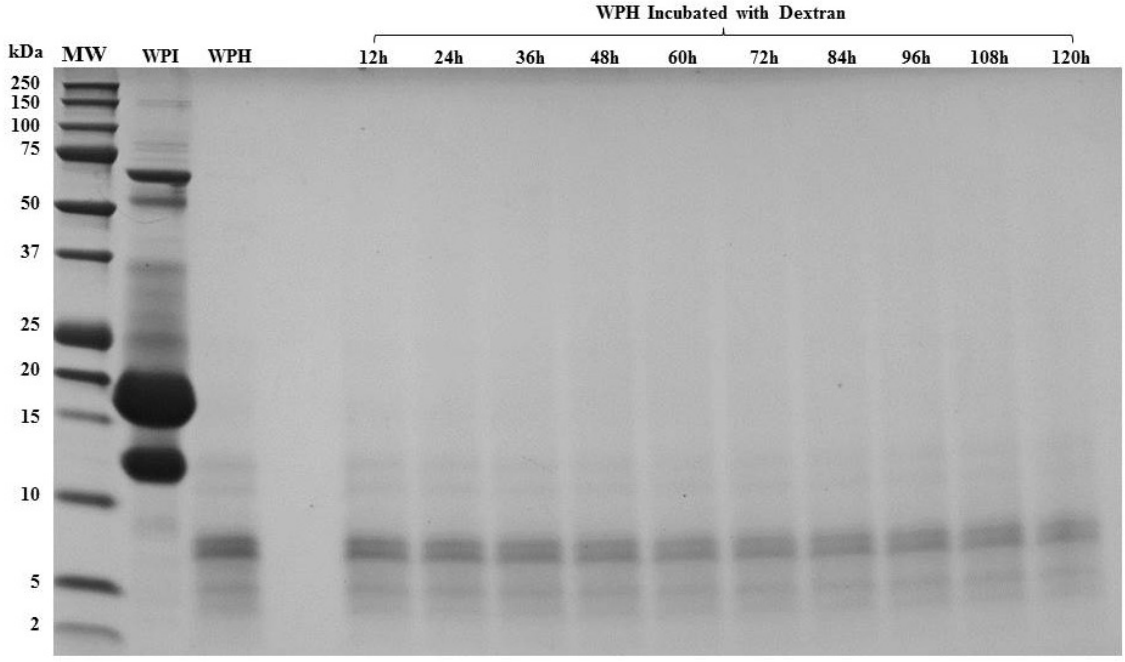

Figure 4. Change in peptide/protein molecular distribution as visualized by Coomassie blue stained SDS-PAGE for WPH incubated with dextran at 60°C for 0-120 h at 49% RH.MW: molecular weight in kDa. Whey protein isolate (WPI) was run as a reference.

Visualization of glycoproteins via SDS-PAGE and glycoprotein staining indicated that glycoproteins were formed and their formation increased with time (Figure 19,

Appendix B). This is indicated by the presence of light smearing in the 50-250 kDa range, absent in the non-incubated WPH lane, which became more apparent over time. The faintness of the smearing suggested that Maillard-glycation was maintained at low-levels throughout the incubation time. This is in accordance with observations made by other researchers, who saw longitudinal smearing after 96h of glycation that was not present in the non-incubated control (Wang & Ismail 2012).

The incubation time selected for further study was 48 h of incubation, as this time point provided moderate Amadori compound formation, with minimal progression to intermediate and final stage MRPs as evidenced by the minimal fluorescent compound formation and browning. Additionally, incubation at this time length resulted in a moderate % loss of free amino groups (21.7%) compared to higher losses (~31%) at longer time periods.

2.4.2 Separation of Free Dextran from Partially Glycated Whey Protein Hydrolysate

The purpose of removing free dextran from the incubated WPH and dextran mixture was three-fold. For one, the presence of free dextran in a final ingredient would allow for the possibility of further initiation of the Maillard reaction beyond desired levels during distribution, storage, and processing, potentially altering the functionality and nutritional quality of the product. Additionally, excess free dextran could contribute to turbidity in fluid applications. Lastly, free dextran could result in undesired textural changes, such as increased viscosity, in some applications.

Preliminary investigation into the separation of free dextran from PGWPH was carried out with hydrophobic interaction chromatography (HIC), which makes use of the reversible insolubility of proteins in high salt solutions. When injecting a non-incubated 1:4 mixture of WPH and dextran, the dextran, all of which was free, eluted within the first 20 min during the 1M ammonium sulfate rinse (Figure 5B). However, problematic was the fact that some protein/peptides were co-eluting with dextran during the 1M ammonium sulfate rinse, as evidenced by the peak that eluted between 10-20 min (Figure 5A). It was hypothesized that this protein/peptide peak, hereafter referred to as the early-eluting protein/peptide peak, was made up of low molecular weight, hydrophilic peptides, which

were not made hydrophobic by the high salt concentration, and so did not effectively bind to the column. A majority of the protein/peptides in WPH were affected by the high salt concentration and eluted with the DDW rinse from 20-35 min (Figure 5A). This is dissimilar to the observations of Wang and Ismail (2012) who used HIC to separate partially glycated whey protein from free dextran. The authors observed that dextran completely eluted during the preliminary 1M ammonium sulfate rinse, while the protein did not elute until the DDW rinse. This is because WPI is comprised of fully intact whey proteins, and so could be effectively made hydrophobic during the high salt rinse, allowing for sufficient separation of protein and free dextran.

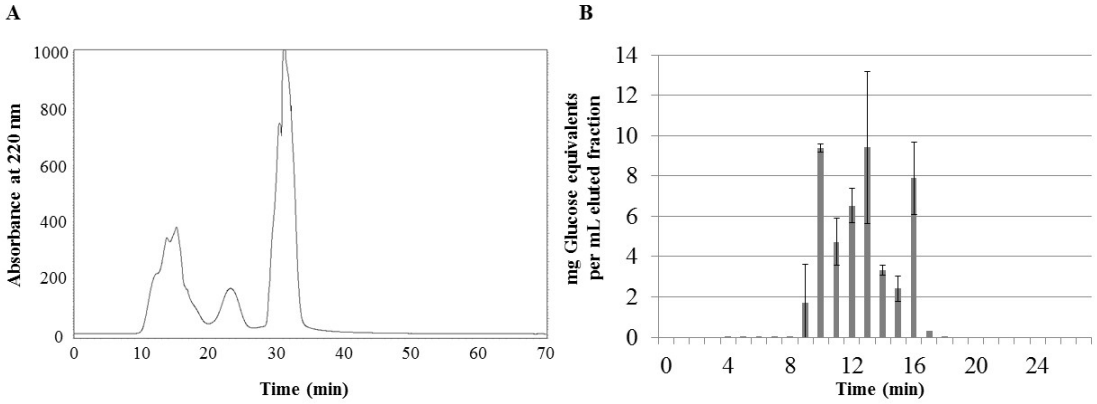

Figure 5. Elution of A) protein and B) carbohydrate of a 4:1 mixture of dextran and WPH. Chromatogram in A shows absorbance at 220 nm, scaled to 1000 absorbance units. Graph B shows mg glucose equivalents per mL of fractions collected every 3 mL (1 min), determined by the phenol-sulfuric acid method (AOAC Method 988.12). Error bars represent standard errors (n=2).

To address the problem of co-elution of dextran with the early-eluting protein peak, hypothesized to be comprised of low molecular weight, hydrophilic peptides, centrifugal filtration using 3 kDa membranes was used to separate peptides < 3 kDa from 10 kDa dextran and >3 kDa peptides and proteins (both glycated and non-glycated). Preliminary testing using a non-incubated 1:4 mixture of WPH and dextran showed that after filtering, the carbohydrate elution pattern of the retentate remained similar to the unfiltered sample (Figure 6, B and D) and most of the dextran appeared to elute within the first 10-20 min during the 1M ammonium sulfate rinse. The peptide peak eluting between 10-20 min, as

determined by absorbance at 220 nm, was slightly reduced in the retentate after filtering compared to the unfiltered sample (Figure 6, A and C). Small peaks (at 10, 20, and 30 min of elution) could be observed in the permeate (Figure 6, E) suggesting that a small amount of peptides were able to pass through the membrane during the filtration, along with only trace free dextran (Figure 6, F).

Testing using WPH incubated with dextran also showed that most of the free dextran appeared to elute within the first 10-20 min during the 1M ammonium sulfate rinse (Figure 7, B and D). However detection of carbohydrate was extended, in low levels, up to 40 min and within the DDW rinse, which was not seen in Figure 6. This additional detected carbohydrate is likely the glycated protein/peptides in WPH incubated with dextran, which elute with the bulk of the protein in the DDW rinse. Only a small amount of peptides were present in the permeate (Figure 7, E), with trace dextran (2.0 mg total) (Figure 7, F).

To increase the concentration of peptides in the permeate, centrifugal filtration was also carried out on WPH incubated with dextran prepared at a 2.0% (w/v) protein concentration, a decrease from the 4.0% (w/v) protein concentration used in the previous experiment. Filtration at this lower protein concentration greatly increased the amount of peptides that passed through the membrane and into the permeate (Figure 8). This proved that high protein concentrations ultimately led to poorer filtration performance as seen in Figure 6E and 7E, most likely due to fouling of the membrane. However, carbohydrate content of the permeate also increased to 58.1% of the dried permeate mass, so a second filtration step was investigated to decrease the carbohydrate content, while maintaining the protein content of the permeate.

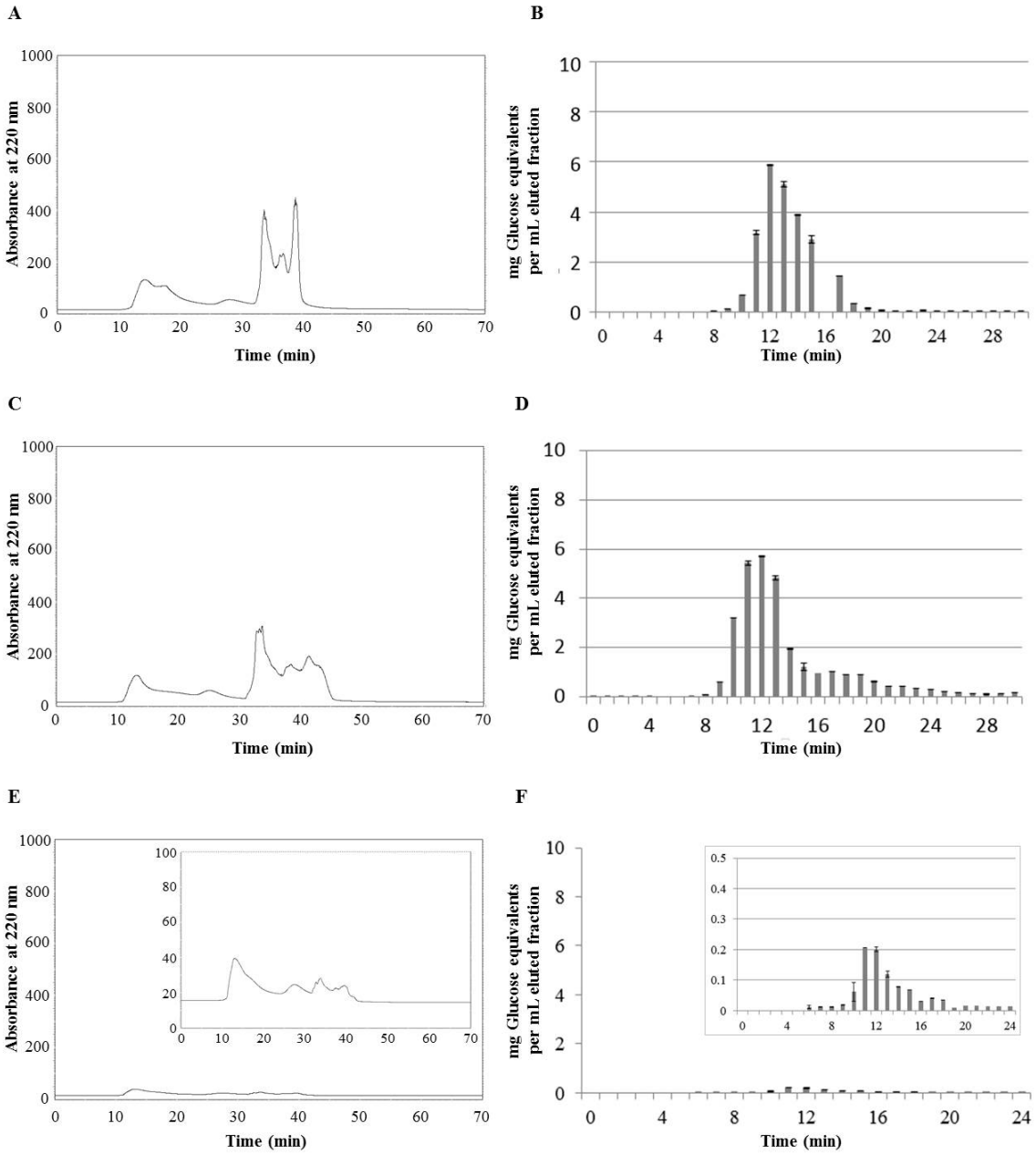

Figure 6. Elution of A) protein and B) carbohydrate of a 4:1 mixture of dextran and WPH; elution of C) protein and D) carbohydrate of the retentate of centrifuge filtration processed 4:1 mixture of dextran and WPH; and elution of E) protein and F) carbohydrate of the permeate of centrifuge filtration processed 4:1 mixture of dextran and WPH. Chromatograms show absorbance at 220 nm, scaled to 1000 absorbance units. Additional chromatogram in E is scaled to 100 absorbance units. Graphs B, D, and F show mg glucose equivalents per mL of fractions collected every 3 mL (1 min), determined by the phenol-sulfuric acid method (AOAC Method 988.12). Error bars represent standard errors (n=2).

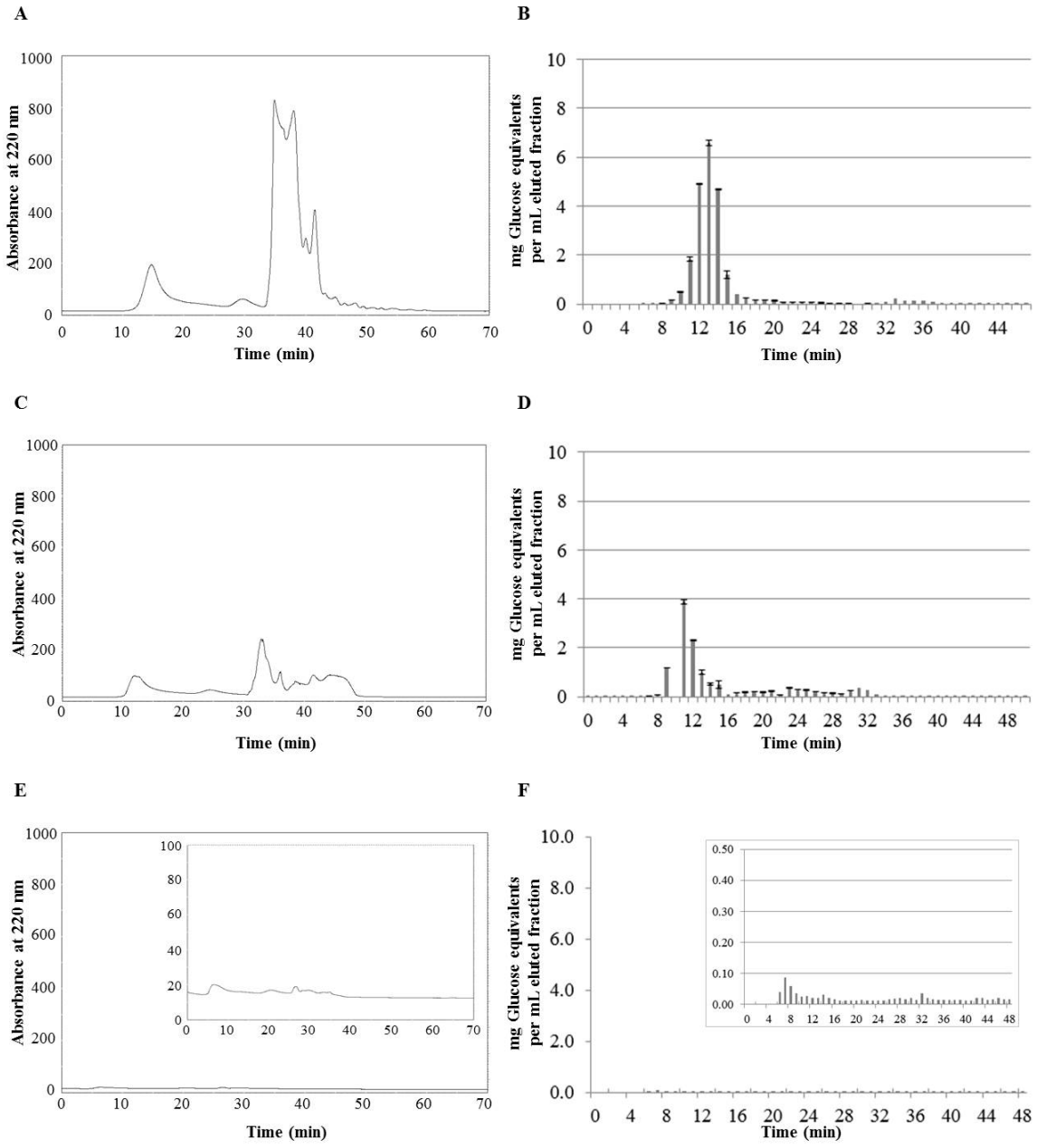

Figure 6. Elution of A) protein and B) carbohydrate of incubated WPH and dextran; elution of C) protein and D) carbohydrate of the retentate of centrifuge filtration processed incubated WPH and dextran; and elution of E) protein and F) carbohydrate of the permeate of centrifuge filtration processed incubated WPH and dextran. Chromatograms show absorbance at 220 nm, scaled to 1000 absorbance units. Additional chromatogram in E is scaled to 100 absorbance units. Graphs B, D, and F show mg glucose equivalents per mL of fractions collected every 3 mL (1 min), determined by the phenol-sulfuric acid method (AOAC Method 988.12). Error bars represent standard errors (n=2).

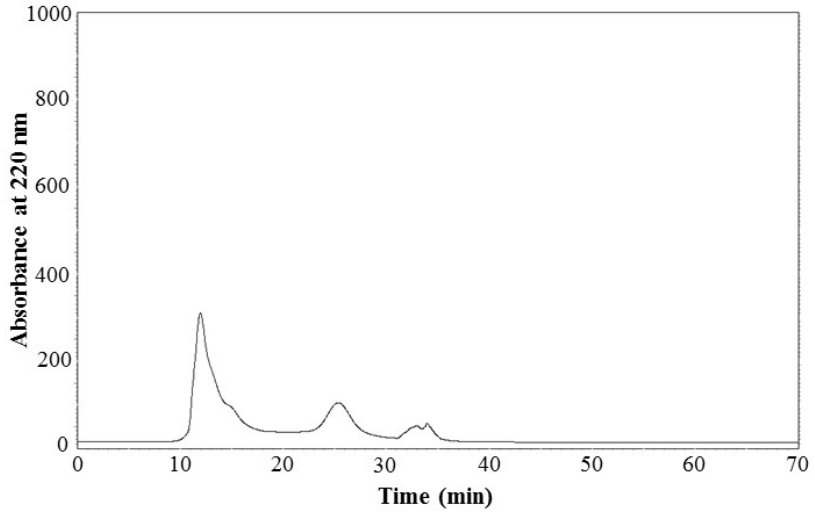

Figure 7. Elution of protein of the permeate of centrifuge filtration processed WPH incubated with dextran (2 mL injection volume, 2.0% protein (w/v)). Chromatogram shows absorbance at 220 nm, scaled to 1000 absorbance units.

To determine the optimal filtration time for a second filtration step, a test was carried out using the permeate from the filtration of WPH incubated with dextran. To test the rate at which protein and dextran were passing through the filter, the sample was centrifuged for 60 min, and aliquots of the permeate was removed for analysis at 10 min intervals to test for protein and carbohydrate content. Carbohydrate pass through was effectively reduced by this second filtration step throughout the 60 minutes of filtration (Figure 9). Protein/peptides passed through the filter continuously over the course of the 60 min of filtration and ~95% recovery of protein/peptides was achieved with 60 min of filtration. So, 60 min was determined to be the appropriate time length for optimal separation of dextran and peptides in the second centrifuge filtration step.

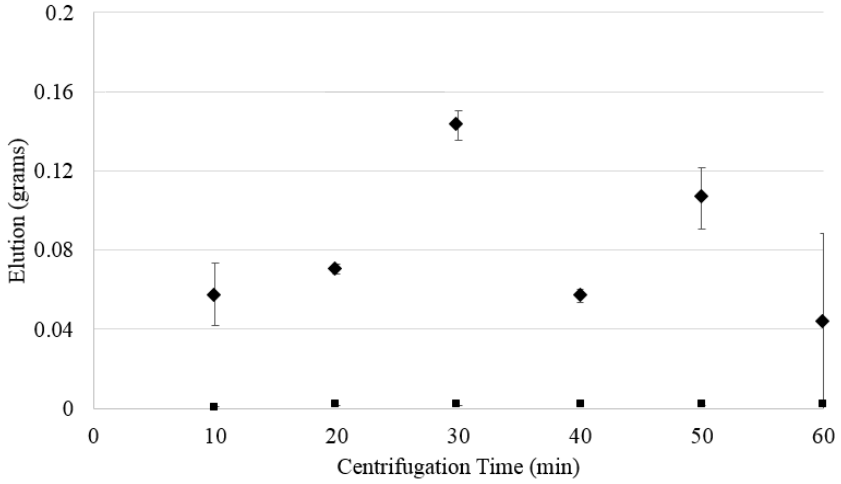

Figure 8. Protein (◆) and carbohydrate (■) contents of centrifuge filtration processed WPH incubated with dextran permeate after 10-60 minutes of a secondary filtration.

The yields after both filtration steps and after HIC separation and desalting during scale-up production is shown in Table 2. The starting mass of WPH incubated with dextran before the first centrifugation step was 126.3g, containing 25.3 g protein. Yields in excess of 80% total mass were maintained through both filtration steps. Losses during the filtration steps were likely due to losses during transfers, and sample fouling on the filtration membranes. The yield after HIC separation and desalting is presented in terms of protein yield, as loss of carbohydrate mass was expected and desired with HIC separation. After HIC separation and desalting, 9.6g of the original 25.3 g protein was retained. However, the issues leading to losses during HIC separation have been identified, and the separation is currently being further optimized to increase yield and maximize retention of peptides.

One issue resulting in sample loss during HIC separation and desalting was the use of a 10 kDa molecular weight cut-off membrane for desalting after HIC separation. The membrane was used for efficient removal of ammonium salts from the column eluent, however, a large portion of WPH was composed of peptides < 10 kDa as can be seen in Figure 4. Therefore, it is likely that most WPH peptides < 10 kDa were inadvertently removed during the desalting step, with exception of those that had been

glycated to 10 kDa dextran during incubation. This removal of non-glycated peptides likely greatly increased the total proportion of glycated peptides in our PGWPH which may lead to significant changes in product functionality, stability, and overall quality.

The second issue resulting in sample loss during removal of free dextran was column fouling. The fouling was discovered after the completion of all separation runs, and is likely the result of hydrophobic proteins/peptides which strongly bind to the hydrophobic column resin. This issue is currently being addressed through the use of several sequential column rinses using water and dilute sodium hydroxide to aid in the elution of hydrophobic peptides.

Table 2. Yields of WPH incubated with dextran after first filtration, second filtration, and after completion of HIC separation and desalting. (Initial Mass: 126.3 g)

	Mass after First Filtration (g)	Mass after Second Filtration (g)	Mass after HIC Separation and Desalting (g)
Retentate	93.7	---	---
Permeate	11.6	8.18	---
Total	105	102	18.1 (9.6g protein)
% Yield	83.4%	80.65%	38% (protein yield)

2.4.3 Characterization of Partially Glycated Whey Protein Hydrolysate

PGWPH was composed of almost 1:1 protein and carbohydrate (Table 3), though it was not confirmed whether all carbohydrate detected was part of glycated protein complexes, or whether some free dextran contamination had occurred. Wang and Ismail (2013) observed that their partially glycated whey protein isolate, produced by incubation of WPI with dextran for 96 h at 60°C and 49% RH and purified using HIC, was comprised of approximately 60% protein and 30% carbohydrate. This could be due, in part, to the increase chemical reactivity and accessibility of free amino groups of WPH compared to WPI. It is also likely that by dialyzing with a 10 kDa membrane, almost all non-glycated

peptides between 3-10 kDa were removed, leaving mostly glycated peptides, and intact proteins (both glycated and non-glycated), thus elevating the ratio of carbohydrate to protein.

Table 3. Moisture, Protein, and Carbohydrate Composition of PGWPH and WPH.

	% Moisture ± SE[a]	% Protein ± SE[a]	% Carbohydrate ± SE[a]
PGWPH	2.85 ± 0.22	53.02 ± 0.07	49.8 ± 1.412
WPH[b]	5.2 ± 0.3	89.6 ± 0.3	< 1.0

[a]Standard Error [b] From manufacturer's specifications

Only 3.37 mg of furosine per g protein was detected in PGWPH (Table 4) (equivalent to 33.4 mg furosine per g lysine; as the WPH used in these experiments had 10.1 g lysine/ 100 g protein). Given that furosine represents ~43.4% of the total Amadori compounds formed under the conditions used for hydrolysis (Krause et al. 2003), the calculated % lysine blockage was 4.4% (on a molar basis). This represents a fairly minimal reduction in protein nutritional quality. Other researchers, including Jimenez-Castano et al. (2005a) saw much greater increases in blocked lysine, specifically 30% lysine blockage in β-lactoglobulin after 48h incubation with dextran at 60°C and 0.44 a_w, however they used a higher ratio of protein to dextran (1:2 compared to 1:4), which may explain the higher lysine blockage observed.

Table 4. Furosine content and Percent Lysine Blockage of WPH and PGWPH.

	Furosine ± SE[a] (mg per g protein)	Furosine ± SE[a] (mg per g lysine)	% Lysine Blockage± SE[a] (on molar basis)
PGWPH	3.37 ± 0.67	33.4 ± 2.6	4.4 ± 0.34
WPH	0.67 ± 0.05	6.64 ± 0.52	0.88 ± 0.068

[a]Standard Error

There was no significant difference in the digestibility of WPH (91.65 ± 1.219%) and PGWPH (89.393 ± 2.99%), which were both very high. Post digestion, the chromatographic peaks representing the hydrolyzed peptides of PGWPH were comparable to those of digested WPH (Figure 20, Appendix C), indicating no change in enzyme hydrolysis due to glycation. The high digestibility of PGWPH along with its minimal lysine blockage indicates that partial glycation of WPH does not decrease its high nutritional quality.

2.5 Conclusions

Maillard-induced glycation of WPH was carried out under controlled conditions to optimize Amadori compound formation, while minimizing progression to intermediate and final stage MRPs including florescent compounds and brown pigments. WPH incubated with dextran for 48h was chosen for further study due to its moderate formation of Amadori compounds, low browning and fluorescent compound formation, and only moderate reduction in free amino groups. Comprehensive testing was carried out to devise a procedure for reducing the quantity of free dextran in glycated whey protein hydrolysate. The final separation protocol removed free dextran from glycated and non-glycated protein and peptides with moderate success using a 2-step membrane filtration and hydrophobic interaction chromatography (HIC). However, desalting with a 10 kDa membrane likely removed non-glycated peptides < 10 kDa. The final composition of glycated whey protein hydrolysate was approximately 1:1 protein to carbohydrate. Purified glycated whey protein hydrolysate displayed minimal blockage of the essential amino acid lysine, and no significant decrease in digestibility. Overall, this work has shown that partial Maillard glycation can be induced and controlled to low-levels in whey protein hydrolysate, producing a value-added product with maintained nutritional quality, and potentially enhanced functionality and stability. Further testing will investigate whether Maillard-glycation of WPH will improve its stability during storage, specifically with regard to moisture-induced protein/peptide aggregation.

3. Physiochemical Changes of a Partially-Glycated Whey Protein Hydrolysate during Accelerated Shelf-life Testing

3.1 Overview

A controlled accelerated shelf-life study of PGWPH and WPH was carried out at 45°C and 31%/65% relative humidity (RH) for up to 28 days to evaluate the effects of glycation on the powder stability. Specifically, the effects of partial glycation of WPH on production of new initial-stage Maillard reaction products (MRPs), progression of MRPs generated upon production of PGWPH to intermediate and advanced stages, and moisture-induced protein/peptide aggregation were monitored. The moisture-sorption isotherm generated for PGWPH indicated that it had a monolayer moisture value (m_0) of 5.92 g water/100 g solids at a corresponding water activity (a_w) of 0.35 at 25°C. Greatest change in color, and formation of fluorescent compounds was observed for the samples stored at 65% RH, with PGWPH experiencing the most change, likely due to progression of the MRPs, generated upon production of PGWPH, to advanced stages of the reaction. Formation of insoluble aggregates and changes in surface hydrophobicity index were not detected for either PGWPH or WPH under the storage conditions studied. However, when heated at 80°C for 30 min at pH 4.5 PGWPH remained soluble while WPH lost over 60% of its solubility. This work demonstrated that partially-glycated whey protein hydrolysate experiences minimal deteriorative reactions during controlled storage, specifically below 65% RH, which is promising for the advancement of protein glycation as a novel protein-enhancement technique.

3.2 Introduction

Whey protein hydrolysates are value-added products that are experiencing a rapid increase in usage and market volume (Affertsholt 2010). Whey protein hydrolysates have several benefits over traditional isolates including the presence of bioactive peptides (FitzGerald & Meisel 2003), increased digestibility (Sinha et al. 2007), and improved functionality (Mullally et al. 1994; Foegeding et al. 2002; Creusot & Gruppen 2007), and thermal stability (Foegeding et al. 2002). However, a challenge with the commercial use of hydrolysates in food products is that their increased reactivity in many deteriorative reactions, including moisture-induced protein/peptide aggregation and the Maillard reaction, can lead to decreased shelf-life and sensory quality (Netto et al. 1998). To allow for continued increases in usage of protein hydrolysate in food formulations, it is important that their stability challenges be addressed.

Moisture-induced protein/peptide aggregation refers to the clustering of protein molecules and the formation of generally irreversible aggregates, primarily through intermolecular hydrophobic interactions and disulfide-bond formation (Costantino et al. 1994; Weiss et al. 2009). The formation of protein/peptide aggregates can lead to decreases in functionality, nutritional quality, and processability. Environmental conditions, such as increased temperature, and relative humidity (RH) are known to increase the rate of protein aggregation (W.R. Liu et al. 1990; Zhou & Labuza 2007; Zhou et al. 2008b), which is problematic given the conditions frequently experienced during modern industrial distribution and storage. Additionally, common chemical reactions including the later stages of the Maillard reaction may cause protein polymerization and exacerbate protein aggregation (Zamora & Hidalgo 2005; Zhou et al. 2013).

Typically, the study of moisture-induced protein/peptide aggregation is carried out using storage studies at various temperatures, relative humidities, or other conditions meant to replicate the realities of the product storage experience. One frequently used storage study type is the accelerated shelf-life test (ASLT) which makes use of elevated temperature (typically 45°C) to increase the rate of the reactions being studied, and to allow for a greater visibility of changes that would otherwise have taken much longer to

see at lower temperatures. Moisture-sorption isotherm generation is also frequently carried out when evaluating the stability of products to moisture-induced changes. Greater susceptibility of a product to moisture content and environmental RH changes can lead to substantial challenges with product physical and physiochemical stability.

Several studies have been conducted on the storage stability of protein hydrolysate powders with respect to moisture-induced protein aggregation. For instance, moisture-induced protein aggregation was monitored during storage of egg white protein hydrolysate (Rao & Labuza 2012), soy protein hydrolysate (Gillman 2014) and whey protein hydrolysate (Zhou & Labuza 2007; Zhou et al. 2014). The studies on WPH in particular indicated that formation of insoluble aggregates was an issue at high RH (>70%), and that disulfide interactions, and hydrophobic interactions to a lesser extent, were responsible for the formation of aggregates (Zhou & Labuza 2007; Zhou et al. 2014).

Environmental control is the primary technique currently being used to control and limit protein/peptide aggregation during industrial production, distribution, and storage. However, in recent years researchers have observed that Maillard-induced glycation decreases protein aggregation in certain food systems (Chevalier et al. 2001; Wang & Ismail 2012). It has been hypothesized that Maillard-induced protein glycation may reduce moisture-induced protein/peptide aggregation of protein hydrolysates in a dry powder system. Therefore, it is our objective to assess the ability of partially-glycated whey protein hydrolysate to retard moisture-induced aggregation during an accelerated storage study using challenging environmental conditions.

3.3 Materials and Methods

3.3.1 Materials

All materials are the same as listed in **Section 2.3.1**, with the addition of standard color plates. Standard color plates (yellow, gray, white, pink, blue, and green) were purchased from Hunter Associates Laboratory, Inc. (Fairfax, VA, USA).

3.3.2 Preparation of Partially Glycated Whey Protein Hydrolysate

Controlled and limited Maillard glycation of whey protein hydrolysate and removal of excess free dextran from PGWPH was carried out as outlined in **Section 2.3.2** and **Section 2.3.4.3**.

3.3.3 Moisture Sorption Isotherm Generation

A moisture sorption isotherm for PGWPH was created using an Aqualab Vapor Sorption Analyzer (VSA) (Decagon Devices, Inc., Pullman, OR, USA). A sample (500 mg) was loaded into a stainless steel analysis cup. The current moisture content (as determined by Karl Fischer) was recorded to track the change in weight and was used to determine the change in moisture content with the change in relative humidity (RH) to generate the isotherm. A dynamic vapor sorption (DVS) isotherm was carried out by exposing the sample to water activities (a_w) from 0.05 to 0.75 at 0.05 a_w intervals. Only after the weight was stable for 3 reading points (indicating that the sample a_w had reached equilibrium) was the RH increased to the next highest level.

The completion of one DVS isotherm took approximately 6 days. To determine the monolayer value (m_0) and the surface heat constant (C), a Brunauer-Emmett-Teller (BET) isotherm model was created for PGWPH using the **Equations 7-9**. The BET graph was created by plotting $a_w/([1- a_w]$moisture content) on the y-axis and a_w on the x-axis as shown by the BET equation (**Equation 7**). The y-intercept and slope of the trend line were used to calculate the m_0 (**Equation 8**) and C (**Equation 9**). A Guggenheim-Andersen-deBoer (GAB) isotherm model was then created to model the change in moisture content with changes in water activity above 0.55. The GAB graph was created by plotting

a_w/moisture on the y-axis and a_w on the x-axis as shown by the GAB equation (**Equation 10**).

Equation 7

$$\frac{a_w}{[(1-a_w)m]} = \frac{1}{m_0 C} + \left[\frac{(C-1)}{m_0 C}\right] a_w$$

Equation 8

$$\mathrm{m_0} = \frac{1}{\frac{1}{m_0 C} + \left[\frac{(C-1)}{m_0 C}\right]}$$

Equation 9

$$\mathrm{C} = \frac{1}{\frac{1}{m_0 C} + \left[\frac{(C-1)}{m_0 C}\right]} \times \frac{1}{m_0 C}$$

Equation 10

$$\mathrm{m} = \frac{\mathrm{C_1 k m_0 a_w}}{(1-\mathrm{k a_w})(1-\mathrm{k a_w}+\mathrm{C_1 k a_w})}$$

Where:
a_w = water activity
m_0 = monolayer moisture value
k = multilayer constant
C = surface heat constant

3.3.4 Storage Study Experimental Design

To determine extent of aggregation during storage of PGWPH and WPH powders an accelerated shelf-life study was designed. PGWPH and WPH were stored at 2 different water activities (a_w) at 45°C for up to 28 days. Desiccators containing saturated salt slurries of magnesium chloride ($MgCl_2$, 0.31 a_w) and potassium iodide (KI, 0.65 a_w) were used to control the humidity of the storage environment and hence the a_w of the samples. A storage temperature of 45°C was chosen to simulate accelerated shelf-life testing. By increasing the storage temperature the rates of chemicals reactions were increased, allowing for a greater possible observation of changes than could otherwise be seen in the same period of

time with a lower temperature. Samples to be checked for color were stored in glass Petri dishes in thin, flat layers to allow for maximum water adsorption. Samples to be collected for analysis at various pre-determined time points (specifically, after 3, 5, 7, 10, 14, 21, and 28 days of storage) were stored in glass HPLC vials, without caps, with the same starting mass of 50 mg protein. Samples were removed at specific times, and stored at -20°C until analysis.

3.3.4.1 Color Analysis by Chroma Meter

The change in color of PGWPH and WPH during storage was measured using a Chroma Meter CR-211 (Minolta Camera Co., Osaka, Japan) tri-stimulus color analyzer. The meter was first calibrated using standard white CR-221 calibration plate, and six color standards (yellow, gray, white, pink, blue, and green). Each sample was measured in triplicate, following the manufacturer's instructions in the absolute measuring mode, using the CIE (International Commission on Illumination) 1976 L*a*b color space system. The L^* values (lightness) extend between 0 for black and 100 for white; the positive a^* values are red/magenta and the negative a^* values are green; the positive b^* values are yellow and the negative b^* values blue. The a^* and b^* values do not have a limited scale in contrast to the lightness scale, which is bound by 0 and 100. To measure the overall change in color over time, the total color difference (ΔE) was calculated (**Equation 11**). The ΔE takes into account the differences between the L^*, a^*, and b^* values from a stored sample and its respective day 0 control.

Equation 11

$$\Delta E = \sqrt{\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2}}$$

Where:
ΔE = total color difference
ΔL^* = change in L^* value from day 0 to day of measurement
Δa^* = change in a^* value from day 0 to day of measurement
Δb^* = change in b^* value from day 0 to day of measurement

Reaction rate constants were determined where possible using linear regressions of the color values plotted versus storage time in days for each color measurement (L*, a*,

b*, and ΔE). Goodness of fit (R^2) was used to indicate how well the linear model fit the experimental data. Model-predicted color values at 28 days were determined using the linear regression equation for each color measurement. Confidence Intervals (95%) were used to indicate significant differences between storage %RH and sample type.

3.3.4.2 Determination of Fluorescent Compounds

The change in fluorescent compound formation in PGWPH and WPH during storage was monitored as outlined in **Section 2.3.3.2**. PGWPH and WPH samples from each pre-determined time point were analyzed in triplicate. The fluorescence intensity (FI) of all incubated samples were adjusted by subtracting the FI values of their respective day 0 controls, as in **Equation 13**. Reaction rate constants were determined where possible using linear regressions as in **Section 3.3.4.1**.

Equation 12:

$$\%\ Fluorescence\ Intensity\ (FI)/g\ protein = \left(\frac{\frac{Emi_{Stored\ Sample}}{g\ Protein} - \frac{Emi_{Day\ 0\ Control}}{g\ Protein}}{Emi_{5\ ppm\ Quinine\ Sulfate}} \right)$$

Where:
Emi = emission reading at 460 nm (bandwidth 30 nm)

3.3.4.3 Loss of Free Amino Groups

The change in free amino group content of PGWPH and WPH during storage was determined by the o-phthalaldehyde (OPA) method as outlined in **Section 2.3.3.3**. PGWPH and WPH samples from each pre-determined time point were analyzed in triplicate. The % remaining free amino group content over storage time was determined using **Equation 13**.

Equation 13:

$$\%\ Remaining\ Free\ Amino\ Group\ Content = \frac{\%\ Free\ Amino\ Groups\ of\ Stored\ Sample}{\%\ Free\ Amino\ Groups\ of\ Day\ 0\ Control} \times 100$$

3.3.4.4 Evaluation of Peptide Profile

The change in peptide distribution and size of PGWPH and WPH during storage was visualized with tris-tricine SDS-PAGE under reducing conditions as outlined in **Section 2.3.3.4**. PGWPH and WPH samples from each pre-determined time point were analyzed in triplicate. Gels were stained with Coomassie blue stain as outlined in **Section 2.3.3.4.1**.

3.3.4.5 Determination of Surface Hydrophobicity

The change in surface hydrophobicity of PGWPH and WPH during storage was determined as described by Kato and Nakai (1980) and Sava and Planken (2005), with modifications. PGWPH and WPH samples from each pre-determined time point were analyzed in triplicate. For each, 0.005-0.050% protein solutions (w/v) were prepared in citric acid phosphate buffer (0.017 M citric acid, 0.165 M sodium phosphate, pH 7). A 0.025% solution (w/v) of 8-Anilino-1-napthalenesulfonic acid ammonium salt (ANS) in phosphate buffer (80mM K_2HPO_4, 19.8 mM KH_2PO_4, pH 7.4) was made fresh at the time of analysis. A 200 µL aliquot of samples were loaded into opaque, white polystyrene microplate and the relative fluorescence index (RFI) was measured at an excitation of 400 nm (bandwidth 30 nm), emission of 460 nm (bandwidth 40 nm), and a gain of 25 with a microplate reader (Biotek, Winooski, VT). A 10 µL aliquot of ANS solution was then added to each well and the plates were incubated at 23°C in the dark for 15 minutes before reading the RFI a second time. The surface hydrophobicity was determined by calculating the Net RFI of each sample by subtracting the RFI of samples before addition of ANS from the RFI of samples after addition of ANS. A plot of the Net RFI for each sample at various protein concentrations was generated, along with a linear regression trend-line for the plot. The initial slope (S_0) of the linear regression is equal to the protein surface hydrophobicity index.

3.3.4.6 Quantitative Determination of Solubility

The change in water solubility of PGWPH and WPH during storage was performed as outlined by Zhou (2008a), and Gillman (2014) with modifications. PGWPH and WPH samples from each pre-determined time point were analyzed in triplicate. For each, a 2.5% protein solution (w/v) was prepared in DDW, in triplicate. Solubilized samples were vortexed and then shaken for one hour. Afterwards, 1 mL of each sample was removed to determine protein content by Dumas using the LECO® Nitrogen Analyzer as in **Section 2.3.5.1**. The remaining dispersions were then centrifuged at 15,682 x g for 10 min and the supernatants were removed for protein content analysis. To determine the percent protein solubility for each sample, **Equation 14** was used.

Equation 14

$$\%\ \text{Protein Solubility} = \frac{(\text{original protein content} - \text{supernatant protein content})}{\text{original protein content}} \times 100$$

The change in solubility of PGWPH and WPH at pH 4.5 and 3.4, after heat treatment at 80°C for 30 min was also assessed. PGWPH and WPH samples from each pre-determined time point were analyzed in triplicate. For each, a 2.5% protein solution (w/v) was prepared in DDW. Solubilized samples were vortexed and then shaken for one hour. Afterwards, samples were adjusted to pH 4.5 or 3.4 using 3N HCl and heated in an 80°C water bath for 30 min. After heating, 1 mL of each sample was removed to determine protein content by Dumas using the LECO® Nitrogen Analyzer as in **Section 2.3.5.1**. The remaining dispersions were then centrifuged at 15,682 x g for 10 min and the supernatants were removed for protein content analysis. To determine the percent protein solubility for each sample, **Equation 14** was used.

3.3.5 Statistical Analysis

Analysis of variance (ANOVA) was carried out using IBM SPSS Statistics software version 22.0 for Windows (SPSS, Inc., Chicago, IL, USA). Significant differences

among the respective means were determined when a factor effect or an interaction was found to be significant ($P \leq 0.05$) using the Tukey-Kramer multiple means comparison test. ANOVA tables for Chapter 3 can be found in Appendix F (Tables 13-44).

3.4 Results and Discussion

3.4.1 Moisture Sorption Properties of Partially-Glycated Whey Protein Hydrolysate

Moisture-sorption isotherms give indication of the change in moisture content of a substance with changing water activity (a_w). Isotherms can also be used to calculate other important physiochemical parameters including the monolayer moisture value (m_o) and surface heat constant (C). The m_0 indicates the moisture content at a specific a_w at which moisture is tightly bound to the substance, and is the moisture content that results in the lowest rates of chemical reactions, and thus the greatest stability (Katz and Labuza 1981). The C indicates how tightly water is bound within the substance.

The moisture-sorption isotherm for PGWPH had a sigmoidal, or Type II shape, which is the most common for food products (Figure 10). The BET model indicated an m_0 of 5.9 g water/100 g solids, at a corresponding a_w of 0.35. This m_0 is similar to that observed by researchers of other protein hydrolysate powders at 23°C including soy protein hydrolysate (m_0 6.8 at 0.29 a_w) (Gillman 2014) and whey protein hydrolysate (m_0 6.1, a_w not specified) (Zhou and Labuza 2007). The model also indicated a C of 2.97, which was lower than that observed by researchers of soy protein hydrolysate (C 8.00) (Gillman 2014) and much lower than that of whey protein hydrolysate (C 60.39) (Zhou and Labuza 2007), suggesting that PGWPH binds water less tightly than other non-glycated protein hydrolysates. However, these authors also determined their m_0 and C values using the Guggenheim-Anderson-de Boer (GAB) model, which may be the reason for these differences.

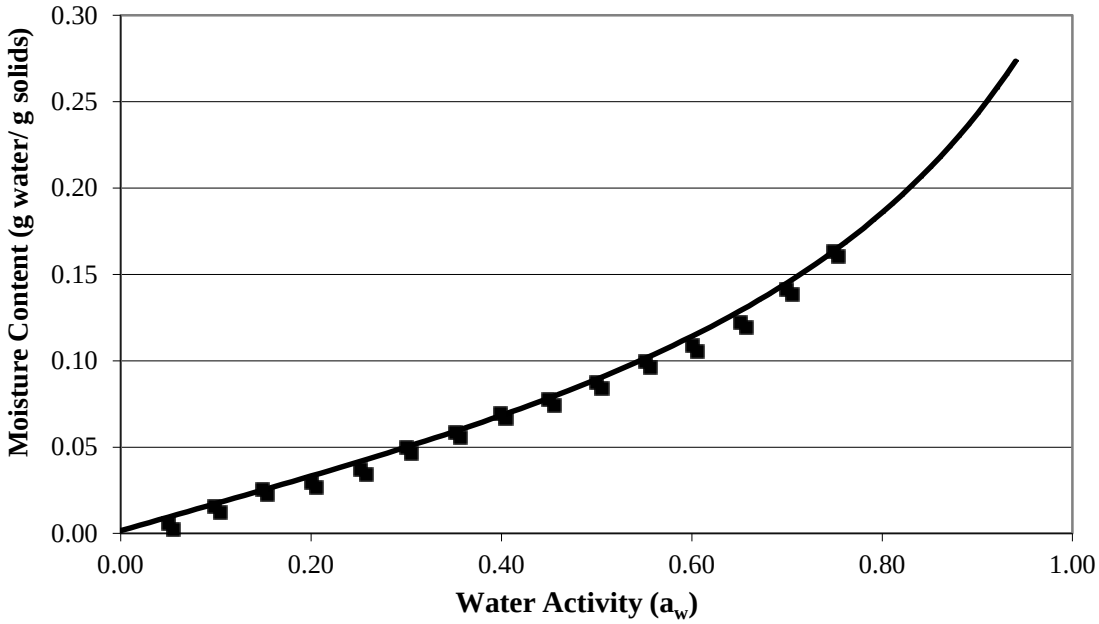

Figure 9. BET/GAB Moisture sorption isotherm of PGWPH at 25°C. Points shown are the points at which equilibrium to the RH were achieved.

The isotherm was also used to indicate RH regions that would be most interesting to investigate during the subsequent study of PGWPH storage stability. The isotherm was used to select 31% and 65% RH for further study of PGWPH storage stability. The RH of 31% was selected as it is very near the m_0, and would approximate PGWPH stability at typical industrial conditions. In contrast, 65% RH was selected as it is sufficiently higher than the m_0 but below the RH at which mold growth would be expected, and so could indicate the stability of PGWPH at conditions of unfavorable RH.

3.4.2 Progression of the Maillard Reaction during Accelerated Shelf-life Testing of PGWPH

Propagation of the Maillard reaction was investigated during accelerated shelf-life testing of PGWPH for several reasons. For one, it is possible that the initial-stage MRPs generated during glycation of PGWPH may progress into intermediate and final stage products. Also, since there is the possibility that PGWPH may contain residual free dextran, new initial-stage Maillard products may be created. It has been observed by other

researchers (Jimenez-Castano et al. 2005; Zhou et al. 2013) that the final and intermediate products formed in the Maillard reaction may contribute to protein/peptide aggregation. And lastly, the Maillard reaction has been known to cause decreases in protein quality via losses of the essential amino acid lysine (Labuza and Warren 1977), and through production of toxic compounds (Brands 2000; Finot et al. 1981), therefore it is important to monitor the reaction during storage.

3.4.2.1 Changes in Total Color and L*, a*, and b* Values during Storage

Color is one of the most basic and common criterions used to determine the acceptability of food ingredients, and so is frequently used as an indicator of quality in shelf-life studies. Color can be used as an indicator of many deteriorative reactions including the Maillard reaction. This is particularly useful as the Maillard reaction is a common mode of quality loss during storage of dry protein powders that contain even small amounts reducing compounds (Dattatreya et al. 2007; Rao and Labuza 2012; Zhou et al. 2014). The system used to measure color of the samples during the study was the L*a*b color space system, which gives indication of a sample's lightness, hue, and total change in color using 4 indices: L*, a*, b*, and ΔE.

The L* dimension of the color space system gives indication of change in lightness of a sample ranging from 0 (black) to 100 (white). PGWPH stored at 65% RH displayed the greatest change in L* value over time. The L* value of PGWPH decreased over time, corresponding to a darkening of the sample (Figure 11). After 28 days, the L* value of PGWPH was significantly different ($P < 0.05$) than at day 0, and was significantly different than all other samples. In contrast, all other samples displayed no significant difference from day 0 to 28, and no significant difference between each other at day 28. The decrease in L* value for PGWPH stored at 65% could be fit with a zero-order model with a reaction rate constant of -0.133 (Table 5). Zero-order models could be constructed for all other samples, but there was a corresponding low goodness of fit due to the minimal changes observed.

A decrease in L* value is characteristic of the Maillard reaction, specifically the intermediate and final stages that involve the production of melanoidins (Rao et al. 2012; Rao and Labuza 2012). It is apparent that PGWPH stored at 65% RH underwent more Maillard reaction during storage. This is plausible, as the Maillard reaction proceeds at faster rates between 0.5-0.8 a_w due to increased molecular mobility of the reactants (Eichner and Karel 1972). Additionally, PGWPH may contain trace free dextran remaining after purification, allowing for initiation of the reaction. Other researchers have observed significant Maillard browning of protein hydrolysates with only trace amounts of reducing sugar when stored at an unfavorable RH (Rao and Labuza 2012; Rao et al. 2012). However, this decrease in lightness may also be due to progression of the initial-stage MRPs, generated during the production of PGWPH, to intermediate and final stage MRPs including melanoidins. The Amadori compound is the first stable intermediate of the Maillard reaction, however, at elevated temperature and RH conditions as tested in this study, it is plausible that these initial compounds would further progress to later stage derivatives.

The reason for the fluctuation of L* values of PGWPH and WPH stored at 31% RH, and WPH stored at 65% RH is likely due to changes in light reflection due to the adsorption of moisture or changes in microstructure of the powders with storage at elevated humidity levels (Rao et al. 2012; Zhou et al. 2014; Gillman 2014). Researchers observed minimal changes in the L* value of WPH when stored below 73% RH and at or below 45°C (Zhou et al. 2014). Changes observed above 73% RH were attributed to changes in protein powder microstructure. However, other researchers attributed the slight changes in L* value to changes in moisture adsorption (Rao et al. 2012; Gillman 2014). The starting moisture content of PGWPH, for example, was 2.94% (w/v, dry basis), which the moisture sorption isotherm predicts would increase to 5.0% at 31% RH and 12.6% at 65% RH. Increase in adsorbed moisture may result in a change in light reflection of a sample, and does not reflect the chemical properties of the sample (Rao et al. 2012).

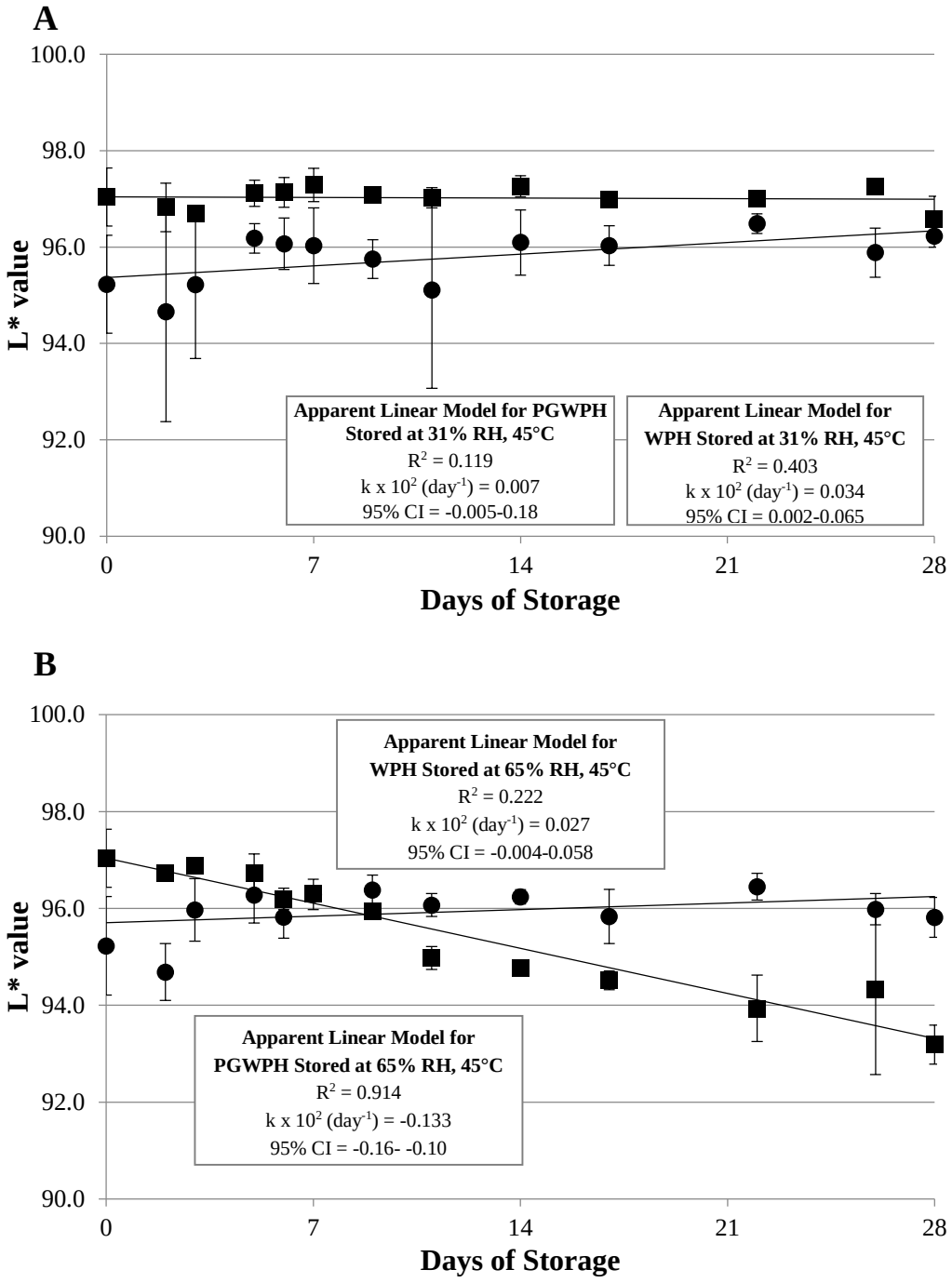

Figure 10. Changes in L* values of PGWPH (■) and WPH (●) during storage at 45°C and 31% (A) and 65% (B) RH. Error bars represent standard error (n=3). R^2 values indicate goodness of fit for each trend-line.

Table 5. Kinetic analysis of zero-order model for the change in L* value as a function of sample type and storage RH at 45°C.

Apparent Linear Model	31% RH		65% RH	
	PGWPH	WPH	PGWPH	WPH
Best fit values				
L^*_{28}	97.1	96.2	93.3	96.4
k x 10^2 (day^{-1})	0.007	0.034	-0.133	0.027
95% CI				
L^*_{28}	97.0-97.4[c*]	95.7-96.8[b]	92.8-93.8[a]	95.8-96.9[b]
k x 10^2 (day^{-1})	-0.005-0.018[b]	0.002-0.065[b]	-0.16- -0.10[a]	-0.004-0.058[b]
bGoodness of fit				
R^2	0.119	0.403	0.914	0.222

L^*_{28}: model predicted values for L* after 28 days of storage
k x 10^2 (day^{-1}): reaction rate constant
[*] Values in the same row with different letters are significantly different ($P \leq 0.05$) by the use of a 95% confidence interval (CI).

The a* and b* dimensions of the color space system indicate a change in hue of a sample, with a* ranging from green (negative values) to red (positive values), and b* ranging from blue (negative values) to yellow (positive values). All samples displayed minimal change in a* value over time (Appendix D). Due to the minimal changes observed, a zero-order model could not be constructed due to poor goodness of fit. PGWPH and WPH stored at 31% RH, and WPH stored at 65% RH became slightly greener, though this was only significant ($P < 0.05$) for WPH stored at 65% RH. PGWPH stored at 65% RH became slightly redder, though the change was not statistically significant. Other researchers observed increases of redness of nonfat dry milk powder stored at 50°C, however, these increases were also very small (Liu and Metzger 2007).

In the b* dimension, all samples became more yellow over time. PGWPH stored at 65% RH displayed the greatest increase in b* value, followed by WPH stored at 65% RH. The samples stored at 31% RH showed only slight increases in b* value over time. This increase in b* value over time with storage of protein powders has been seen by several other researchers (Liu and Metzger 2007; Gillman 2014). However, neither the a*

nor b* values are traditionally used to indicate the Maillard reaction, and so they may reflect other physiochemical changes of the powder such as moisture adsorption.

The total change in color value (ΔE) quantifies the total change in the L*, a*, and b* dimensions of a sample over time, and so gives the best indication of overall color change. The greatest observed total color change (12.4) after 28 days of storage was observed for PGWPH stored at 65% RH (Figure 12). Indeed, PGWPH stored at 65% had significantly the highest reaction rate constant (k) for the zero-order model fit for total color change (Table 5). WPH stored at 65% had the next highest change in total color (5.8) and a significantly ($P < 0.05$) lower reaction rate constant. PGWPH and WPH stored at 31% RH showed significantly ($P < 0.05$) different, though minimal changes in color. Having minimal changes made it difficult to fit a zero-order trend line to WPH stored at 31%, leading to the poor goodness of fit observed ($R^2 = 0.403$). Values shown in Table 5 are model-predicted values for ΔE after 28 days of storage, and so differ slightly from the experimentally observed values.

The highest total change in color was observed in the samples incubated at the higher RH, 65%. This is expected as most chemical reactions, including those that result in changes in color, are increased with higher RH due to increased molecular mobility of reactants and increased water content, which may serve as a reactant or solvent (Labuza 1980; Bell 2007). The primary color change observed for PGWPH stored at 65% RH was a decrease in lightness, likely attributed to initiation or progression of the Maillard reaction. The primary color change observed for WPH stored at 65% RH was an increase in yellowness, which could be due to a variety of factors including moisture adsorption or possibly the Maillard reaction (Liu and Metzger 2007; Gillman 2014). The samples stored at 31% RH showed minimal change in lightness, but slight increases in yellowness. This lack of color change was expected as 31% RH corresponds to a sample water activity of 0.31, which is near the monolayer of PGWPH. At the monolayer chemical reactions rates are at their lowest, and so product stability is maximized (Labuza and Schmidl 1986).

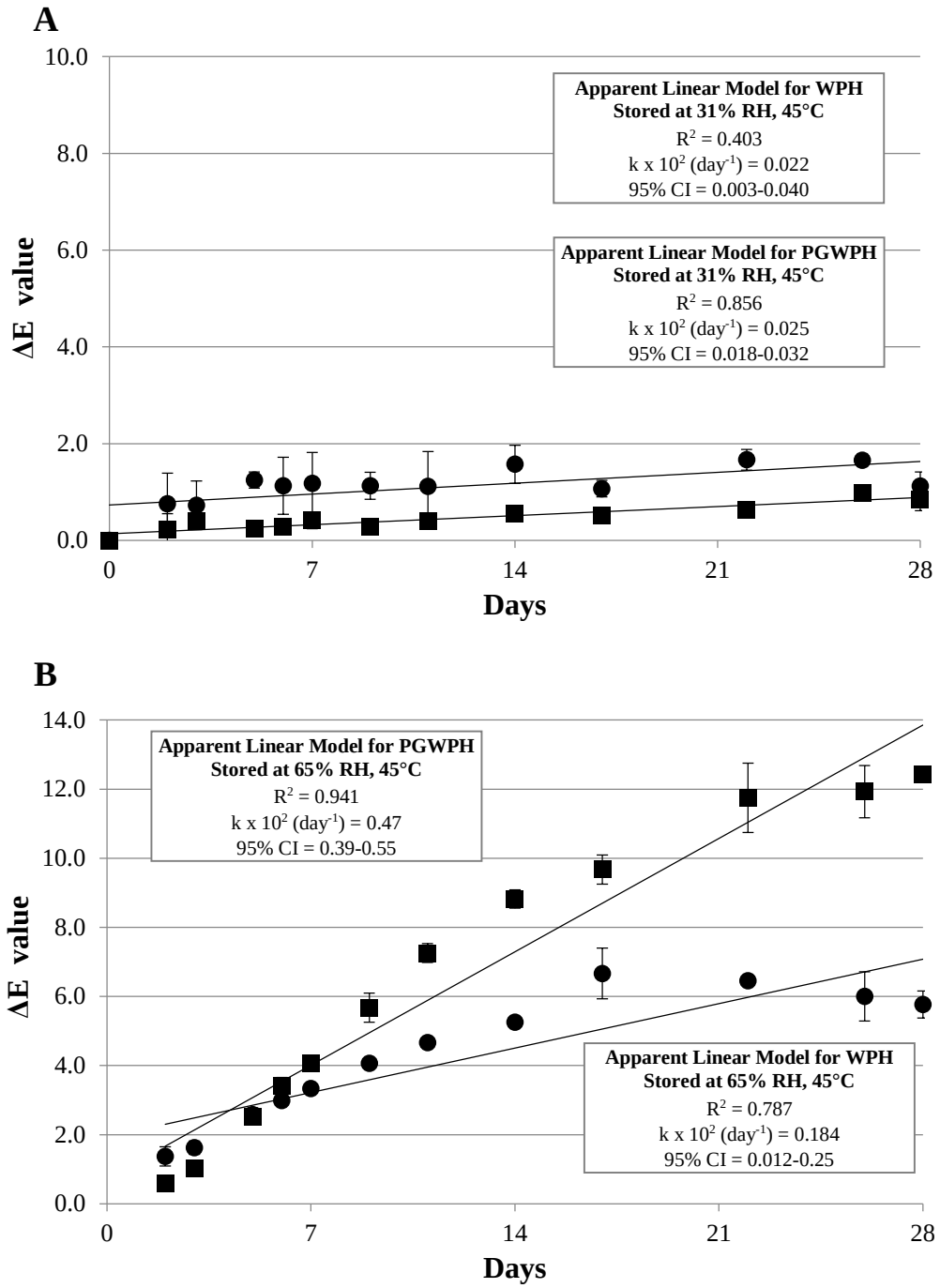

Figure 11. Total color change (ΔE) values of PGWPH (■) and WPH (●) during storage at 45°C and 31% (A) and 65% (B) RH. Error bars represent standard error (n=3). R^2 values indicate goodness of fit for each trend-line.

Table 6. Kinetic analysis of zero-order model for the change in ΔE value as a function of sample type and storage RH at 45°C.

Apparent Linear Model	31% RH		65% RH	
	PGWPH	WPH	PGWPH	WPH
Best fit values				
ΔE_{28}	0.87	1.53	13.9	7.08
k x 10^2 (day^{-1})	0.025	0.022	0.47	0.184
95% CI				
ΔE_{28}	0.74-0.99[a*]	1.20-1.86[b]	12.4-15.3[d]	5.89-8.27[c]
k x 10^2 (day^{-1})	0.018-0.032[a]	0.003-0.040[a]	0.39-0.55[c]	0.12-0.25[b]
Goodness of fit				
R^2	0.856	0.403	0.941	0.787

ΔE_{28}: model predicted values for ΔE after 28 days of storage
k x 10^2 (day^{-1}): reaction rate constant
[*] Values in the same row with different letters are significantly different ($P \leq 0.05$) by the use of a 95% confidence interval (CI).

3.4.2.2 Changes in Fluorescence Intensity during Storage

Determination of change in fluorescence intensity of stored samples was used to indicate progression of the Maillard reaction to the intermediate stage. While color can be used to indicate production of melanoidins in the later stages of the Maillard reaction, the measurement of fluorescence can be a useful indicator of the progression of the Maillard reaction beyond the initial stage, but before melanoidins are detectable (Friedman and Kline 1950; Morales and Boekel 1997).

The samples stored at different RH displayed different rates of fluorescent compound production. PGWPH stored at 65% showed the greatest formation of fluorescent compounds after 28 days (Figure 13), and the greatest rate of fluorescent compound formation when fit with a zero-order model (Table 6). The next highest reaction rate and quantity of fluorescent compounds formed was for WPH stored at 65% RH. PGWPH and WPH stored at 31% RH significantly ($P < 0.05$) differed in the quantity of fluorescent compounds formed, but not in the rate at which they were formed. Both the rate and the quantity of fluorescent compounds formed after 28 days were minimal.

Though production of fluorescent compounds is indicative of more than one chemical reaction including lipid oxidation, in this case it is likely that production of fluorescent compounds was primarily due to progression to the intermediate stages of Maillard reaction, given that the fat content was minimal (<0.5%, by manufacturer's specifications). The high quantity and significantly ($P < 0.05$) higher rate constant observed for PGWPH stored at 65% RH, suggests that at least in part, initial MRPs were able to progress to the intermediate stage of the reaction. However, it is still unclear whether this was due to MRPs newly formed during this storage study, or whether it was progression of the initial-stage MRPs generated during glycation of PGWPH. The moderate concentration of fluorescent compounds formed in WPH stored at 65% RH was also of interest, as it suggests that the Maillard reaction was also occurring, but to a lesser extent, in the protein powder. The WPH used in this study contains less than 1% lactose (by manufacturer's specifications), but due to its small size and consequent high reducing power, even a small concentration of the lactose could result in significant Maillard reaction over time when stored in conditions that promote the reaction, such as high temperature and humidity. Other researchers have also observed increases in fluorescence and Maillard reaction progression during storage of dry protein powders with low reducing compound concentrations (Rao and Labuza 2012; Gillman 2014).

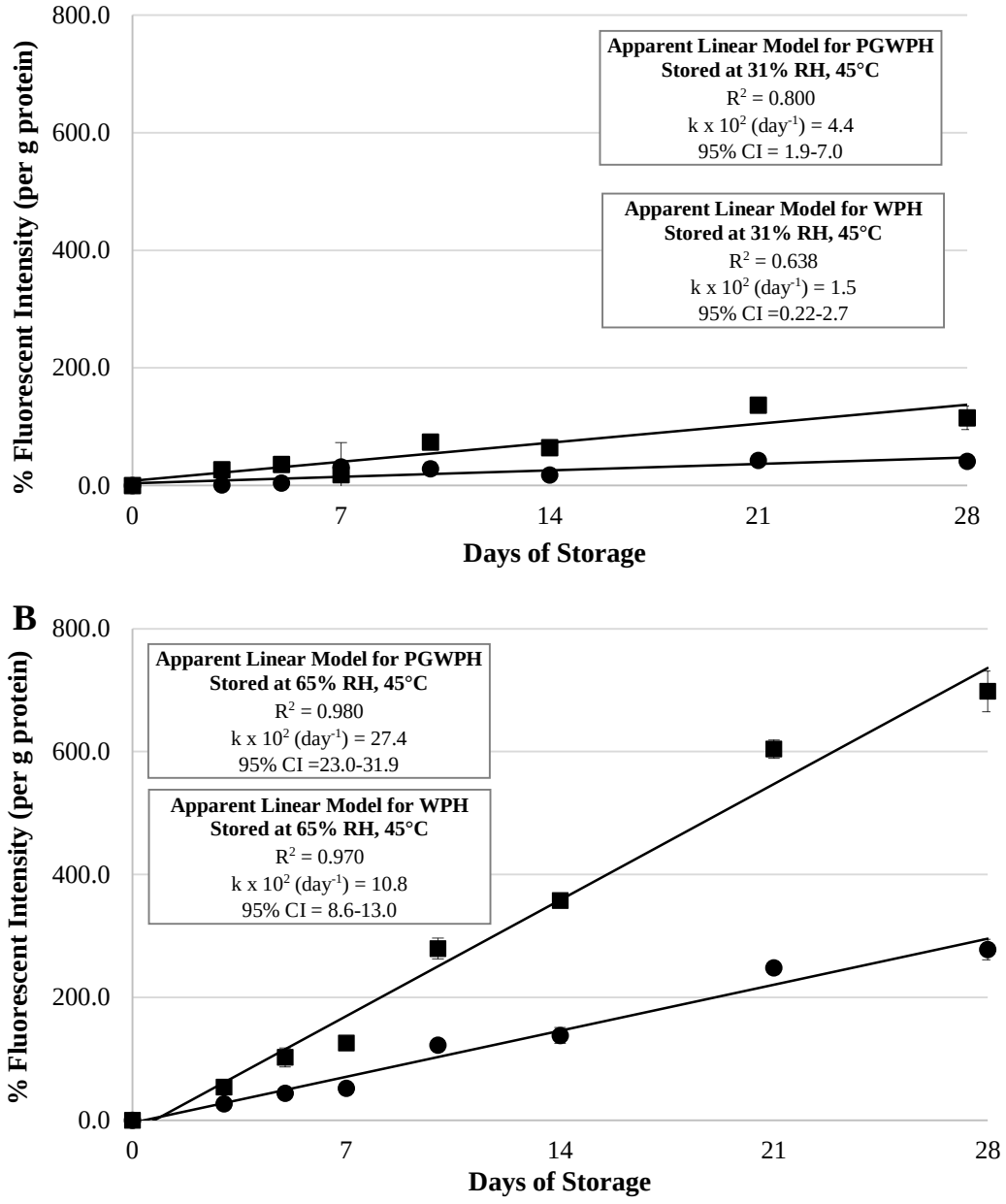

Figure 12. Changes in % fluorescence intensity of PGWPH (■) and WPH (●) during storage at 45°C and 31% (A) and 65% (B) RH. Error bars represent standard error (n=3). R^2 values indicate goodness of fit for each trend-line.

The low quantity and rate of fluorescent compound formation for samples stored at 31% RH is in agreement with the color data presented. Storage at 31% RH for both PGWPH and WPH was close enough to the monolayer of each to result in a minimal Maillard reaction, and to maintain product stability, at least in terms of this particular deteriorative reaction.

Table 7. Kinetic analysis of zero-order model for the change in fluorescence intensity (%FI) value as a function of sample type and storage RH at 45°C.

Apparent Linear Model	31% RH		65% RH	
	PGWPH	WPH	PGWPH	WPH
Best fit values				
% FI_{28}	135.6	46.5	740.8	296.8
k x 10^2 (day^{-1})	4.4	1.5	27.4	10.8
95% CI				
% FI_{28}	90.8-180.3^{b*}	24.2-68.9^{a}	662.1-819.5^{d}	257.7-335.9^{c}
k x 10^2 (day^{-1})	1.9-7.0^{a}	0.22-2.7^{a}	23.0-31.9^{c}	8.6-13.0^{b}
Goodness of fit				
R^2	0.800	0.638	0.980	0.970

k x 10^2 (day^{-1}): reaction rate constant

* Values in the same row with different letters are significantly different ($P \leq 0.05$) by the use of a 95% confidence interval (CI).

3.4.2.3 Change in Free Amino Groups during Storage

To monitor the initiation of the Maillard reaction during storage, free amino group content for each sample was quantified over time. Loss of free amino groups would be expected if new MRPs were formed during storage, and so can be used to indicate initiation of the Maillard reaction before change in color or fluorescence is observed (Warren and Labuza 1977).

All samples stored had only minimal changes in free amino group content over the 28 days of storage (Figure 14). Only WPH stored at 31% RH showed a significant increase in % remaining free amino groups from day 0 to day 28. All other sample types, showed slight increases or decreases over the 28 days of storage, showed no significant difference in free amino content between day 0 and day 28.

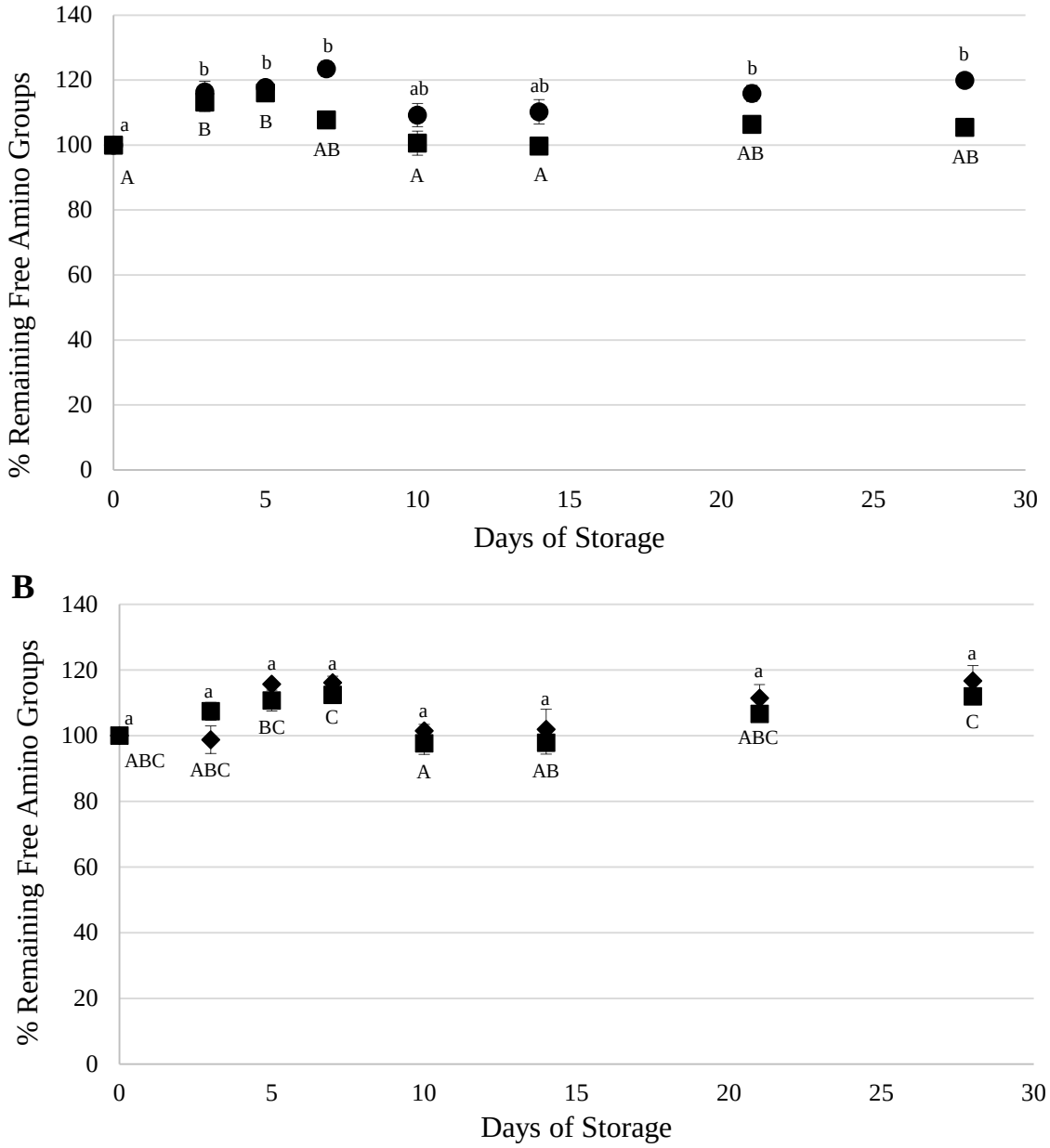

Figure 13. Changes in % remaining free amino group content of PGWPH (■) and WPH (●) during storage at 45°C and 31% (A) and 65% (B) RH. Error bars represent standard error (n=3). Different letters above and below the shapes indicate significant differences across different storage time points according to the Tukey-Kramer multiple means comparison test ($P \leq 0.05$).

The minimal changes in % remaining free amino groups observed over the course of storage could be due to the conditions tested, specifically the RH used and the relatively

short length of the study. Loss of free amino groups in soy protein hydrolysate was most pronounced at 79% RH, over a longer storage period (Gillman 2014); however, at several other RH conditions, no significant change in free amino group content was observed.

There are also several factors that may have contributed to the lack of changes in free amino group content observed. For one, Amadori compound rearrangement products formed during the intermediate stage of the Maillard reaction may regenerate free amino groups through deamidation, fission, or dehydration reactions (Wolf et al., 1977; Labuza & Massaro, 1990; Baisier & Labuza, 1992). Also, microbial proteases could have been introduced that could result in protein cleavage and production of new free terminal amines. However, it may just be that no new Maillard reaction was initiated during the time period of the study, and that changes in color and fluorescence are due to progression of MRPs already present at the beginning of storage rather than generation of new ones. Potentially a combination of all three of these factors may have contributed to the lack of change in % remaining free amino groups during storage of PGWPH and WPH.

3.4.3 Measurement of Protein/Peptide Aggregation during Accelerated Shelf-life Testing of PGWPH

3.4.3.1 Changes in Peptide Profile during Storage

The intermediate and advanced stages of the Maillard reaction can stimulate protein polymerization (Zamora and Hidalgo 2005; Zhou et al. 2013), so as intermediate and advanced MRPs were detected in PGWPH and WPH during storage it was important to monitor aggregation during storage due to this covalent polymerization. Polymerization, and thus change in protein/peptide molecular size, was monitored during storage using SDS-PAGE under reducing conditions. The use of reducing conditions and SDS disrupted disulfide and hydrophobic interactions, respectively, allowing for the observation of any non-disulfide covalent interactions that could have been formed.

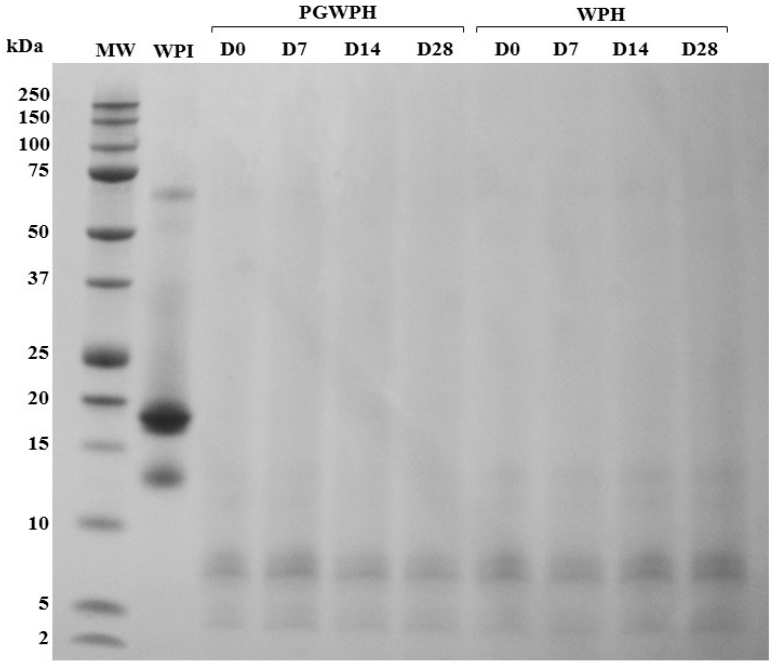

Figure 14. Change in protein/peptide molecular size as determined by Coomassie blue stained SDS-PAGE for PGWPH and WPH stored at 45°C and 65% RH for 7 (D7), 14 (D14), and 28 (D28) days along with non-incubated controls (D0). MW: molecular weight in kDa. Whey protein isolate (WPI) was run as a reference.

Despite the presence of intermediate and advanced stage MRPs during storage, minimal polymerization was visible of PGWPH and WPH stored at 31% RH (Figure 23, Appendix E) or 65% RH (Figure 15). Polymerization could result in new high molecular weight bands at the top of the gel, due to the formation of aggregates that are too large to enter the gel or migrate further down the gel. Polymerization may also result in smearing and band darkening near the top of the gel due to formation of aggregates of varied size, along with a consequent lightening of the low molecular weight bands near the bottom of the gel. Researchers who used SDS-PAGE to investigate the mechanisms of protein aggregation including the effects of hydrophobic interactions, disulfide bonds, and Maillard-induced polymerization during storage of soy protein hydrolysate were similarly unable to detect insoluble aggregates at 59% RH and 45°C after 28 and 77 days (Gillman 2014). It appears that under the RH conditions and time length studied, Maillard-induced formation of insoluble protein/peptide aggregates did not occur at detectable levels.

3.4.3.2 Changes in Surface Hydrophobicity during Storage

As hydrophobic interactions are one of the primary mechanisms of protein/peptide aggregation (Costantino et al. 1994), the surface hydrophobicity index of PGWPH and WPH stored samples was determined. A decrease in surface hydrophobicity index would be expected if proteins/peptides were aggregating based upon hydrophobic interactions, as less hydrophobic regions would be available for the hydrophobic probe used to bind.

For the most part, however, neither PGWPH nor WPH showed any significant decrease in surface hydrophobicity over storage time, or at either of the RH conditions (Figure 16). This observation suggests that if protein/peptide aggregation did occur, hydrophobic interactions were not a major contributor. Both WPH and PGWPH experienced slight decreases in surface hydrophobicity with storage, particularly when stored at 65% RH, however, these differences were, for the most part, not statistically significant.

It was hypothesized that glycation might reduce protein/peptide aggregation during storage due to the various physical and chemical changes that occur with glycation. Several researchers observed a decrease in surface hydrophobicity of glycated protein (Nacka et al. 1998; Mu et al. 2006; Wang and Ismail 2012), which was attributed to the blockage of hydrophobic regions upon glycation, and increased surface hydrophilicity via increased net negative charge and attachment of hydrophilic carbohydrate-moieties. However, researchers also observed an increase in surface hydrophobicity of casein-glucose and milk protein-lactose conjugates, which was attributed to an increase protein unfolding due to loss of tertiary structure upon glycation, and to the conditions (temperature, a_w) used to initiate glycation (Hiller and Lorenzen 2010). Thus, the effects of protein glycation on surface hydrophobicity may depend on the protein and carbohydrate source, among other factors.

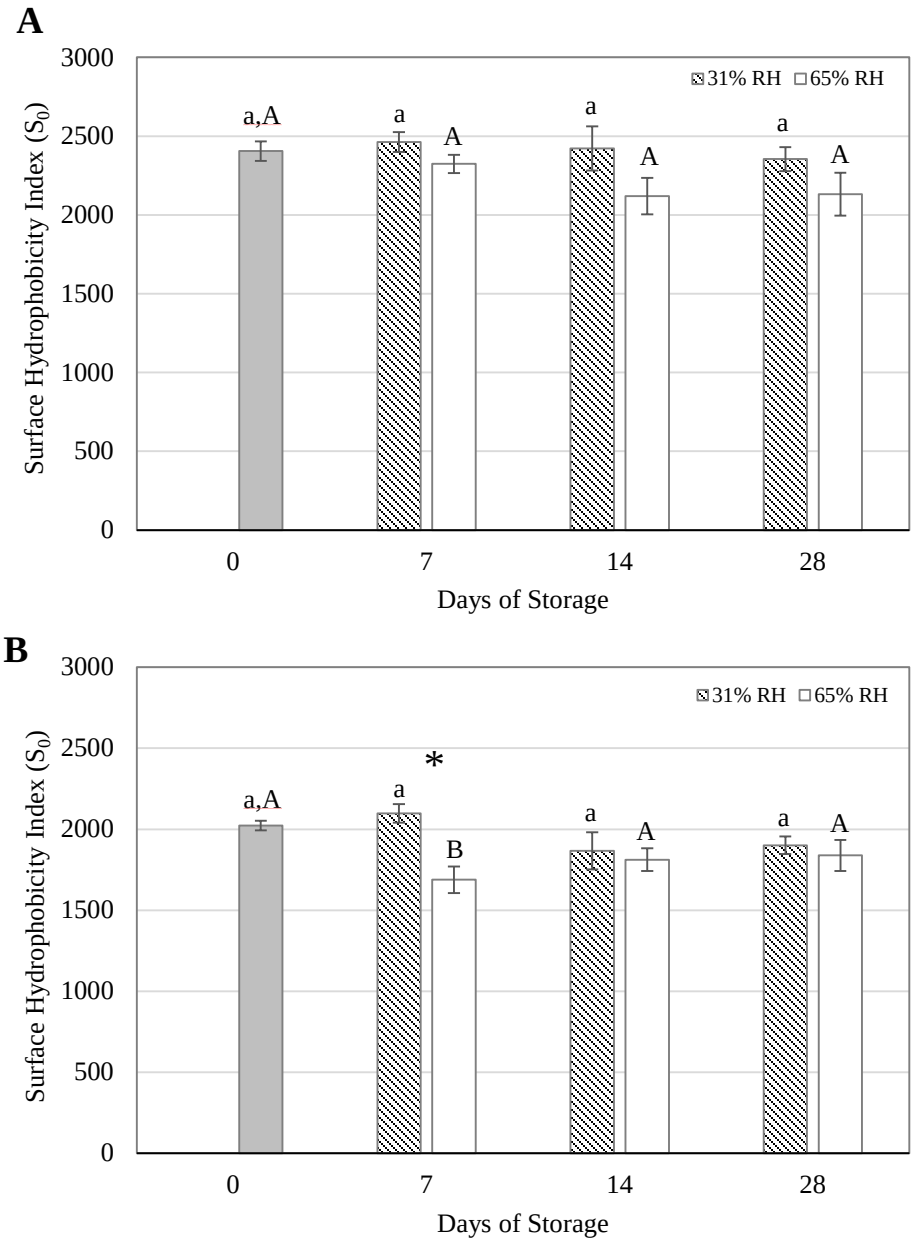

Figure 16. Changes in surface hydrophobicity (S0) of PGWPH (A) and WPH (B) during storage at 31% and 65% RH and 45°C. Error bars represent standard error (n=3). Different letters above the bars indicate significant differences across different storage time points and within each %RH condition according to the Tukey-Kramer multiple means comparison test ($P \leq 0.05$). An asterisk above bars of a time point indicates a significant difference within each % RH condition according to the Tukey-Kramer multiple means comparison test ($P \leq 0.05$).

3.4.3.3 Changes in Solubility and Thermal Stability during Storage

To quantify insoluble aggregation during storage, solubility of PGWPH and WPH was measured under several conditions. A reduction in solubility between day 0 and day 28 would be expected if insoluble aggregates were formed.

For the most part, no significant changes in solubility over time were observed for any of the samples or conditions tested (Figure 17). Both WPH and PGWPH displayed excellent solubility in water, approximately 100%, after 28 days of storage. However, after a 30 minute heat treatment at 80°C and pH 4.5 (near the isoelectric point of whey protein) the solubility of WPH was markedly reduced to below 40%, while solubility was maintained at approximately 100% for PGWPH. Enhanced solubility at pH close to the isoelectric point has previously been observed by other researchers who have glycated intact whey proteins (Wang and Ismail 2012), and so it is promising to see that this property is maintained upon glycation and throughout extended storage of whey protein hydrolysates. After a similar heat treatment at pH 3.4, PGWPH and WPH again maintained 100% of their solubility, which was not affected significantly by storage under any of the conditions studied.

It was initially hypothesized that a change in solubility might be observed in the stored samples, particularly at 65% RH due to increased protein/peptide mobility and potential for formation of intermolecular covalent or noncovalent bonds as seen by other researchers (Zhou and Labuza 2007; Gillman 2014). It must be noted that protein solubility in this study was measured at 2.5% protein concentration, which may have been too dilute to detect changes in solubility for whey protein, which displays excellent solubility ordinarily. However, the combined results of the peptide profile, surface hydrophobicity index, and solubility measurements suggest that aggregation did not occur under the RH conditions and time span studied. Other researchers investigating the storage stability of a similar WPH to what was used in this study only noted a significant decrease in solubility at 45°C over 2 weeks when stored at RH in excess of 70% (Zhou and Labuza 2007). These authors found no formation of insoluble aggregates for intact WPI upon storage at 45°C and over a wide range of RH (11-85%) for up to 2 weeks, but they found high formation

of insoluble aggregates (>50% solubility loss) in WPH when stored at RH in excess of 70%.

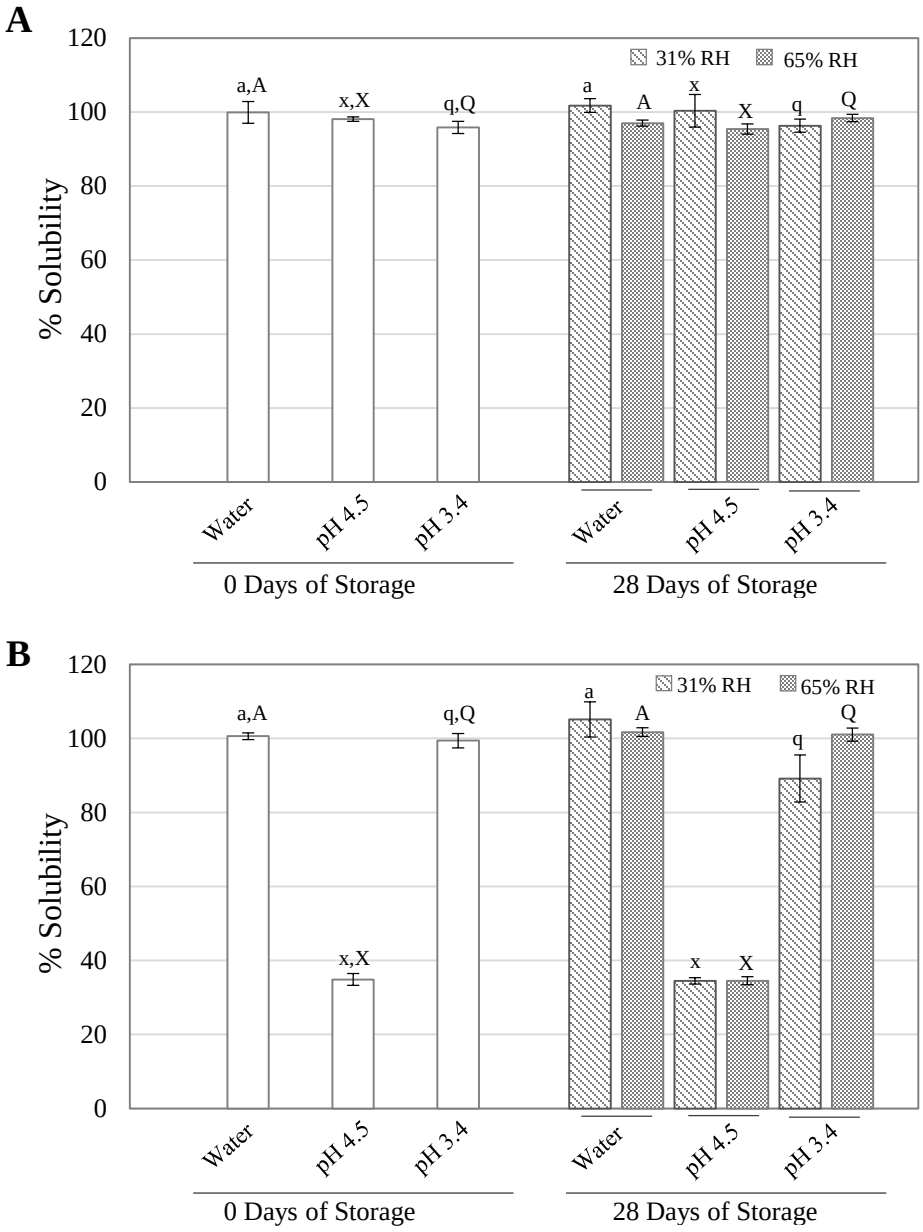

Figure 17. Solubility after 0 and 28 days of storage at 45°C and at 31% and 65% RH of: PGWPH (A) and WPH (B) in water and at pH 4.5 and 3.4 after heat treatment. Error bars represent standard error (n=3). Different letters above the bars indicate significant differences in solubility at a specific pH and % RH between day 0 and 28 according to the Tukey-Kramer multiple means comparison test ($P \leq 0.05$).

When the authors investigated the mechanisms of solubility loss, they found that disulfide interactions were the main contributor to the observed loss, with hydrophobic interactions also playing a small part. This suggests that aggregation is still a problem for WPH powders at severe temperature and RH conditions, and glycation may still show promise as a technique to reduce moisture-induced protein/peptide aggregation since it is able to sterically, or otherwise limit the formation of intermolecular disulfide interactions. Therefore, further storage studies must be conducted at more severe RH conditions for longer periods of time followed by testing of solubility and aggregation at higher protein concentrations to fully understand the effects of protein glycation on aggregation of whey protein hydrolysates.

3.5 Conclusions

A controlled study of PGWPH and WPH stored at 45°C and 31% /65% RH for up to 28 days was carried out to evaluate the effects of partial glycation of WPH on production of new MRPs, progression of the initial-stage MRPs generated upon production of PGWPH, and moisture-induced protein/peptide aggregation. Greatest change in color, and formation of fluorescent compounds was observed for the samples stored at 65% RH, with PGWPH experiencing the most change, likely due to progression of the initial-stage MRPs generated upon production of PGWPH to advanced stages of the reaction. Formation of insoluble aggregates, or changes in surface hydrophobicity index could not be detected at the conditions studied. Further studies must be done to fully understand the effects of protein glycation on aggregation of whey protein hydrolysates. However, this work shows that partially-glycated products of WPH in particular, experience minimal deteriorative reactions during controlled storage, specifically below 65% RH and 45°C, which is promising for the advancement of protein glycation as a novel protein-enhancement technique.

4. Overall Conclusions, Implications, and Recommendations

This work has shown that low-level Maillard-induced glycation of WPH can be achieved under the conditions studied without progression to the advanced stages of the Maillard reaction. It also showed that partial glycation of WPH has minimal effect on the nutritional quality of the protein. This maintenance of nutritional quality is vital if Maillard-glycation is to have wider application in the production of value-added products.

This study also showed that the use of membrane filtration and HIC adequately removed free dextran allowing for the production of glycated whey protein hydrolysate. However, as the process was cumbersome and resulted in mediocre protein yields a more efficient process must be developed to save time and inputs, while increasing product yield and limiting waste streams, to best fit industrial demands. It is also important that this improved separation process retain as many peptides as possible, as peptides are primarily responsible for the enhanced health and functional properties of protein hydrolysates. It is vital that the procedure developed for removal of free dextran be designed with industrial feasibility in mind if Maillard-glycation is to be used for the production of real-world ingredients. Industrial feasibility necessitate limited waste streams, high throughput, and efficient processes. Separation protocol improvement is currently in the works, and will address each of these issues.

The findings of this study also showed that deteriorative reactions including progression of MRPs to advanced stages and moisture-induced protein/peptide aggregation of glycated whey protein hydrolysate were minimal during storage at 31% RH, near the monolayer of PGWPH. This finding affirms the importance of storage at or near the monolayer for protein hydrolysate powders. On the other hand, this work also showed that progression of initial-stage MRPs, generated upon production of PGWPH, to advanced stages can occur when environmental RH is high. This is important to note, and must be taken into consideration in future studies and in the development of protein-glycation technology for the production of commercial ingredients. As high environmental RH may occur in the real world due to non-ideal distribution or storage conditions both for the industrial ingredient, and as the finished consumer product, it is important that glycated

proteins retain their nutritional and functional quality during storage if this technology is to make the transition from bench-top to industrial-scale production.

Finally, this study highlighted the excellent solubility at various pH conditions including the isoelectric point of whey protein and high resilience of glycated whey protein hydrolysate to environmental conditions, as no moisture-induced aggregation was observed under the storage conditions and time period studied. This enhanced stability suggests that whey protein is a promising candidate for future protein-glycation work and has a great potential for industrial application. However, as previous research has indicated that the formation of insoluble aggregates is exacerbated with protein hydrolysis when stored at higher RH, glycation may still show promise as a technique to reduce moisture-induced protein/peptide aggregation. Further studies at higher RH and over longer periods of time must be done to fully understand the effects of protein glycation on aggregation of whey protein hydrolysates.

This study has laid the ground-work for future studies on the industrial feasibility of glycation, the application of Maillard glycation to other protein hydrolysates for greater functionality improvements, and the stability of protein hydrolysates during storage. With this work, the collective knowledge on Maillard-glycation and stability of hydrolysates is expanded.

Comprehensive Bibliography

Adhikari, HR. 1973. Fluorescent products in a glucose-glycine browning reaction. *Journal of Food Science 38*: 486–488.

Affertsholt, T. 2009. International Whey Market Overview. In 3A Business Consulting (ed.), *Annual Conference of the American Dairy Products Institute*. Chicago, IL, USA.

Affertsholt, T. 2010. The World Market for Dairy Bioactive Ingredients 2009-2012. In 3A Business Consulting (ed.), *12th Annual Dairy Ingredients Symposium*. San Fancisco, CA, USA.

Akhtar, M and Dickinson, E. 2003. Emulsifying properties of whey protein–dextran conjugates at low pH and different salt concentrations. *Colloids and Surfaces B: Biointerfaces 31*(1-4): 125–132.

Akhtar, M and Dickinson, E. 2007. Whey protein–maltodextrin conjugates as emulsifying agents: an alternative to gum arabic. *Food Hydrocolloids 21*(4): 607–616.

Alder-Nissen, J. 1986. *Enzymatic Hydrolysis of Food Proteins*. New York City, NY, USA: Elsevier Applied Science Publishers.

Althouse, PJ, Dinakar, P and Kilara, A. 1995. Screening of Proteolytic Enzymes to Enhance Foaming of Whey Protein Isolates. *Journal of Food Science 60*(5): 1110–1112.

Alting, AC, Hamer, RJ, G, de KC and Visschers, RW. 2000. Formation of disulfide bonds in acid-induced gels of preheated whey protein isolate. *Journal of Agricultural and Food Chemistry 48*(10): 5001–5007.

Ames, JM. 1992. The Maillard reaction. In B. J. F. Hudson (ed.), *Biochemistry of Food Proteins*. London, U.K.: Elsevier Applied Science.

Anderson, GH and Moore, SE. 2004. Dietary Proteins in the Regulation of Food Intake and Body Weight in Humans. *The Journal of Nutrition 134*: 974–979.

Baisier, WM and Labuza, TP. 1992. Maillard browning kinetics in a liquid model system. *Journal of Agricultural and Food Chemistry 40*(5): 707–713.

Boirie, Y, Dangin, M, Gachon, Pi, Vasson, M-P, Maubois, J-L and Beaufrere, B. 1997. Slow and fast dietary proteins differently modulate postprandial protein accretion. *Proceedings of the National Academy of Sciences 94*: 14930–14935.

Brands, CM, Alink, GM, van Boekel, M a and Jongen, WM. 2000. Mutagenicity of heated sugar-casein systems: effect of the Maillard reaction. *Journal of Agricultural and Food Chemistry 48*(6): 2271–5.

Bryant, CM and McClements, DJ. 2000. Influence of NaCl and CaCl2 on cold-set gelation of heat-denatured whey protein. *Journal of Food Science 65*(5): 801–804.

Burton, HS, McWeeny, DJ and Biltcliffe, DO. 1963. Non-enzymic browning development of chromophores in the glucose-glycine and sucrose-glycine systems. *Journal of Food Science 28*(6): 631–639.

Calligaris, S, Manzocco, L, Anese, M and Nicoli, MC. 2004. Effect of heat-treatment on the antioxidant and pro-oxidant activity of milk. *International Dairy Journal 14*: 421–427.

Caric, M. 1993. Concentrated and Dried Dairy Milk Products. In Y. H. Hui (ed.), *Dairy Science and Technology Handbook: Vol. 2. Product Manufacturing*. New York: VCH Publishers.

Castilho, RF, Meinicke, AR, Almeida, AM, Hermeslima, M and Vercesi, AE. 1994. Oxidative damage of mitochondria induced by Fe (II) citrate is potentiated by Ca 2+ and includes lipid peroxidation and alterations in membrane proteins. *Archives of Biochemistry and Biophysics 308*(1): 158–163.

Cheison, SC, Wang, Z and Xu, SY. 2007a. Preparation of whey protein hydrolysates using a single- and two-stage enzymatic membrane reactor and their immunological and antioxidant properties: Characterization by multivariate data analysis. *Journal of Agricultural and Food Chemistry 55*: 3896–3904.

Cheison, SC, Wang, Z and Xu, SY. 2007b. Use of response surface methodology to optimise the hydrolysis of whey protein isolate in a tangential flow filter membrane reactor. *Journal of Food Engineering 80*: 1134–1145.

Chevalier, F, Chobert, J, Dalgalarrondo, M and Haertle, T. 2001. Characterization of the Maillard reaction products of B-lactoglobulin glucosylated in mild conditions. *Journal of Food Biochemistry 25*: 33–55.

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

Clemente, A. 2001. Enzymatic protein hydrolysates in human nutrition. *Trends in Food Science & Technology 11*(2000): 254–262.

Corzo-Martinez, M, Moreno, FJ, Olano, A and Villamiel, M. 2008. Structural characterization of bovine b-lactoglobulin-galactose/tagatose Maillard complexes by electrophoretic, chromatographic, and spectroscopic methods. *Journal of Agricultural and Food Chemistry* (56): 4244–4252.

Costantino, HR, Langer, R and Klibanov, AM. 1994. Moisture-induced aggregation of lyophilized insulin. *Pharmaceutical Research 11*(1): 21–29.

Creusot, N and Gruppen, H. 2007. Enzyme-induced aggregation and gelation of proteins. *Biotechnology Advances 25*(6): 597–601.

Creusot, N and Gruppen, H. 2008. Hydrolysis of whey protein isolate with Bacillus licheniformis protease: aggregating capacities of peptide fractions. *Journal of Agricultural and Food Chemistry 56*(21): 10332–9.

Creusot, N, Gruppen, H, van Koningsveld, G a., de Kruif, CG and Voragen, AGJ. 2006. Peptide–peptide and protein–peptide interactions in mixtures of whey protein isolate and whey protein isolate hydrolysates. *International Dairy Journal 16*(8): 840–849.

Damodaran, S. 1994. Structure-Function Relationship of Food Proteins. In N. Hettiarachchy & G. Ziegler (eds.), *Protein Functionality in Food Systems*. CRC Press.

Danquah, M and Agyei, D. 2012. Pharmaceutical applications of bioactive peptides. *OA Biotechnology 1*(2): 1–7.

De Wit, JN. 1990. Thermal stability and functionality of whey proteins. *Journal of Dairy Science 73*(12): 3602–3612.

Dryáková, A, Pihlanto, A, Marnila, P, Čurda, L and Korhonen, HJT. 2010. Antioxidant properties of whey protein hydrolysates as measured by three methods. *European Food Research and Technology 230*(6): 865–874.

Eichner, K and Karel, M. 1971. The influence of water content and water activity on the sugar-amino browning reaction in model systems under various conditions. *Journal of Agricultural and Food Chemistry 20*(2): 218–223.

Elwood, PC, Givens, DI, Beswick, AD, Fehily, AM, Pickering, JE and Gallacher, J. 2008. The survival advantage of milk and dairy consumption: an overview of evidence from cohort studies of vascular diseases, diabetes and cancer. *Journal of the American College of Nutrition 27*(6): 723S–34S.

Elwood, PC, Pickering, JE, Ian Givens, D and Gallacher, JE. 2010. The consumption of milk and dairy foods and the incidence of vascular disease and diabetes: An overview of the evidence. *Lipids 45*: 925–939.

Engberink, MF, Hendriksen, MAH, Schouten, EG, van Rooij, FJA, Hofman, A, Witteman, JCM and Geleijnse, JM. 2009. Inverse association between dairy intake and hypertension: The Rotterdam Study. *American Journal of Clinical Nutrition 89*(C): 1877–1883.

Erbersdobler, HF, Brandt, A, Scharrer, E and Wangenheim, B. 1981. Transport and metabolism studies with fructose amino acids. *Progress in Food and Nutrition Science 5*: 257e–263e.

Esmaillzadeh, A and Azadbakht, L. 2010. Dairy consumption and circulating levels of inflammatory markers among Iranian women. *Public health nutrition 13*(9): 1395–1402.

Finot, P. 1990. *The Maillard reaction in food processing, human nutrition and physiology*. Springer Science & Business Media.

Finot, PA and Magnenat, E. 1981. Metabolic transit of early and advanced Maillard products. *Progress in Food and Nutrition Science*.

Fischer, K. 1935. A new method for the analytical determination of the water content of liquids and solids. *Angew Chem 48*(394): 24.

FitzGerald, RJ and Meisel, H. 2003. Milk Protein Hydrolysates and Bioactive Peptides. In P. F. Fox & P. L. H. McSweeney (eds.), *Advanced Dairy Chemistry: Vol. 1. Proteins* (3rd ed.). New York: Kluwer Academic/Plenum Publishers.

Foegeding, EA, Davis, JP, Doucet, D and McGuffey, MK. 2002. Advances in modifying and understanding whey protein functionality. *Trends in Food Science & Technology 13*(5): 151–159.

Fox, PF. 2003. Milk Proteins: General and Historical Aspects. In P. F. Fox & P. L. H. McSweeney (eds.), *Advanced Dairy Chemistry: Vol. 1. Proteins* (3rd ed.). New York: Kluwer Academic/Plenum Publishers.

Frost & Sullivan. 2013. *Market overview of the global protein ingredients market*.

Funtenberger, S, Dumay, E and Cheftel, JC. 1997. High pressure promotes b-lactoglobulin aggregation through SH/S-S interchange reactions. *Journal of Agricultural and Food Chemistry* (45): 912–921.

Gardner, HW. 1979. Lipid hydroperoxide reactivity with proteins and amino acids: A review. *Journal of Agricultural and Food Chemistry 27*(2): 220–229.

Gillman, L. 2014. The Impact of Moisture-Induced Aggregation of Soy Protein Hydrolysate during Storage on Product and Nutritional Quality. University of Minnesota.

Goodno, CC, Swaisgood, HE and Catignani, GL. 1981. A fluorimetric assay for available lysine in proteins. *Analytical Biochemistry 115*: 203–211.

Groleau, PE, Gauthier, SF and Pouliot, Y. 2003. Effect of residual chymotryptic activity in a trypsin preparation on peptide aggregation in a β-lactoglobulin hydrolysate. *International Dairy Journal 13*(11): 887–895.

Guadix, A, Camacho, F and Guadix, EM. 2006. Production of whey protein hydrolysates with reduced allergenicity in a stable membrane reactor. *Journal of Food Engineering 72*(4): 398–405.

Hall, WL, Millward, DJ, Long, SJ and Morgan, LM. 2003. Casein and whey exert different effects on plasma amino acid profiles, gastrointestinal hormone secretion and appetite. *The British Journal of Nutrition 89*(2): 239–48.

Halton, TL and Hu, FB. 2004. The effects of high protein diets on thermogenesis, satiety and weight loss: a critical review. *Journal of the American College of Nutrition 23*(5): 373–385.

Hattori, M, Ogino, A, Nakai, H and Takahashi, K. 1997. Functional Improvement of -Lactoglobulin by Conjugating with Alginate Lyase-Lysate 703–708.

Havea, P, Singh, H and Creamer, LK. 2002. Heat-induced aggregation of whey proteins: comparison of cheese wpc with acid wpc and relevance of mineral composition. *Journal of Agricultural and Food Chemistry* (50): 4674–4681.

Hiller, B and Lorenzen, PC. 2010. Functional properties of milk proteins as affected by Maillard reaction induced oligomerisation. *Food Research International 43*(4): 1155–1166.

Hodge, JE. 1953. Chemistry of Browning Reactions in Model Systems. *Journal of Agricultural and Food Chemistry 1*(15): 928–943.

Hopton, JD, Swan, CJ and Trimm, DL. 1989. Liquid-phase oxidation of thiols to disulfides. *Advanced Chemistry Series 75*: 216–224.

Horowitz, M, Edelbroek, MAL, Wishart, JM and Straathof, JW. 1993. Relationship between oral glucose tolerance and gastric emptying in normal healthy subjects. *Diabetologia 36*: 857–862.

Huang, XL, Catignani, GL and Swaisgood, HE. 1999. Modification of rheological properties of whey protein isolates by limited proteolysis. *Nahrung/Food 43*(2): 79–85.

Hui, YH (ed.). 1993. *Dairy Science and Technology Handbook*. New York City, USA: VCH Publishers.

Huncharek, M, Muscat, J and Kupelnick, B. 2009. Colorectal cancer risk and dietary intake of calcium, vitamin D, and dairy products: a meta-analysis of 26,335 cases from 60 observational studies. *Nutrition and Cancer 61*(1): 47–69.

Hurley, WL. 2003. Immunoglobulins in Mammary Secretions. In P. F. Fox & P. L. H. McSweeney (eds.), *Advanced Dairy Chemistry: Vol. 1. Proteins* (3rd ed.). New York: Kluwer Academic/Plenum Publishers.

Ismail, B and Gu, Z. 2010. *Whey Protein Hydrolysates : Current Knowledge and Challenges*. Saint Paul, MN, USA.

Jimenez-Castano, L, Lopezfandino, R, Olano, A and Villamiel, M. 2005. Study on β-lactoglobulin glycosylation with dextran: effect on solubility and heat stability. *Food Chemistry 93*(4): 689–695.

Jiménez-Castaño, L, Villamiel, M and López-Fandiño, R. 2007. Glycosylation of individual whey proteins by Maillard reaction using dextran of different molecular mass. *Food Hydrocolloids 21*(3): 433–443.

Jiménez-Castaño, L, Villamiel, M, Martín-Álvarez, PJ, Olano, A and López-Fandiño, R. 2005. Effect of the dry-heating conditions on the glycosylation of β-lactoglobulin with dextran through the Maillard reaction. *Food Hydrocolloids 19*(5): 831–837.

Ju, ZY and Kilara, A. 1998. Textural properties of cold-set gels induced from heat-denatured whey protein isolates. *Journal of Food Science 63*(2): 288–292.

Ju, ZY, Otte, J, Madsen, JS and Qvist, KB. 1995. Effects of Limited Proteolysis on Gelation and Gel Properties of Whey Protein Isolate. *Journal of Dairy Science 78*(10): 2119–2128.

Kato, A and Nakai, S. 1980. Hydrophobicity determined by a fluoresence probe method and its correlation with surface properties of proteins. *Biochimica et Biophysica Acta 624*: 13–20.

Kearsley, MW. 1978. The control of hygroscopicity, browning and fermentation in glucose syrups. *International Journal of Food Science & Technology 13*(4): 339–348.

Kelly, ALL, O'Connell, JEE and Fox, PFF. 2003. Manufacture and Properties of Milk Powders. In P. F. Fox & P. L. H. McSweeney (eds.), *Advanced Dairy Chemistry: Vol. 1. Proteins* (3rd ed., Vol. 1). New York: Kluwer Academic/Plenum Publishers.

Kikugawa, K and Beppu, M. 1987. Involvement of lipid oxidation products in the formation of fluorescent and cross-linked proteins. *Chemistry and Physics of Lipids 44*(2): 277–296.

Kilara, A. 1994. Whey Protein Functionality. In N. Hettiarachchy & G. Ziegler (eds.), *Protein Functionality in Food Systems*. CRC Press.

Kitts, DD and Weiler, K. 2003. Bioactive proteins and peptides from food sources. Applications of bioprocesses used in isolation and recovery. *Current Pharmaceutical Design 9*(16): 1309–1323.

Krause, R, Knoll, K and Henle, T. 2003. Studies on the formation of furosine and pyridosine during acid hydrolysis of different Amadori products of lysine. *European Food Research and Technology 216*: 277–283.

Kronman, MJ, Andreotti, R and Vitols, R. 1964. Inter- and intramolecular interactions of a-lactalbumin. II. Aggregation reactions at acid pH. *Biochemistry 3*(8): 1152–1160.

Kuehler, C a. and Stine, CM. 1974. Effect of Enzymatic Hydrolysis on Some Functional Properties of Whey Protein. *Journal of Food Science 39*(2): 379–382.

Labuza, TP and Schmidl, MK. 1986. Advances in the Control of Browning Reactions in Foods. In O. R. Fennema, W.-H. Chang & C.-Y. Lii (eds.), *Role of Chemistry in the Quality of Processed Food*. Westport, Connecticut: Food & Nutrition Press, Inc.

Labuza, TP, Tannenbaum, SR and Karel, M. 1970. Water content and stability of low-moisture and intermediate-moisture foods. *Food Technology*.

Laemmli, UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature 227*(5259): 680–185.

Lahl, WJ and Braun, SD. 1994. Enzymatic production of protein hydrolysates for food use. *Food Technology*.

Lea, CH and Hannan, RS. 1951. Studies of the reaction between proteins and reducing sugars in the dry state. *Biochimica et Biophysica Acta 3*: 313–325.

Levine, H and Slade, L. 1986. A polymer physico-chemical approach to the study of commercial starch hydrolysis products (SHPs). *Carbohydrate Polymers 6*: 213–244.

Li, CP, Enomoto, H, Ohki, S, Ohtomo, H and Aoki, T. 2005. Improvement of functional properties of whey protein isolate through glycation and phosphorylation by dry heating. *Journal of dairy science 88*(12): 4137–45.

Liu, WR, Langer, R and Klibanov, AM. 1990. Moisture-induced aggregation of lyophilized proteins in the solid state. *Biotechnology and Bioengineering 37*: 177–184.

Liu, X, Powers, JR, Swanson, BG, Hill, HH and Clark, S. 2005. Modification of whey protein concentrate hydrophobicity by high hydrostatic pressure. *Innovative Food Science & Emerging Technologies 6*(3): 310–317.

Luhovyy, BL, Akhavan, T and Anderson, GH. 2007. Whey Proteins in the Regulation of Food Intake and Satiety. *Journal of the American College of Nutrition 26*(6): 704S–712S.

MacLeod, SK. 1991. Moisture determination using Karl Fischer titrations. *Analytical Chemistry 63*(10): 557A–566A.

Madsen, JS, Ahmt, TO, Otte, J, Halkier, T and Qvist, KB. 1997. Hydrolysis of B-lactoglobulin by four different proteinases monitored by capillary electrophoresis and high performance liquid chromatography. *International Dairy Journal 7*: 399–409.

Mangino, ME, Liao, YY, Harper, NJ, Morr, CV and Zadow, JG. 1987. Effects of Heat Processing on the Functionality of Whey Protein Concentrates. *Journal of Food Science 52*(6): 1522–1524.

Matiacevich, S and Pilarbuera, M. 2006. A critical evaluation of fluorescence as a potential marker for the Maillard reaction. *Food Chemistry 95*(3): 423–430.

Matoba, T and Hata, T. 1972. Relationship between bitterness of peptides and their chemical structures. *Agricultural and Biological Chemistry 36*(8): 1423–1431.

McMahon, DJ, Adams, SL and McManus, WR. 2009. Hardening of high-protein nutrition bars and sugar/polyol-protein phase separation. *Journal of Food Science 74*(6): E312–21.

Meisel, H. 1997. Biochemical properties of regulatory peptides derived from milk proteins. *Peptide Science 43*(2): 119–128.

Merritt, RJ, Carter, M, Haight, M and Eisenberg, LD. 1990. Whey protein hydrolysate formula for infants with gastrointestinal intolerance to cow milk and soy protein in infant formulas. *Journal of Pediatric Gastroenterology and Nutrition 11*: 78–82.

Mintel. 2013. Performance Food and Drink - US - January 2013 (January).

Mishra, S, Mann, B and Joshi, VK. 2001. Functional improvement of whey protein concentrate on interaction with pectin. *Food Hydrocolloids 15*: 9–15.

Mleko, S and Foegeding, EA. 2000. pH induced aggregation and weak gel formation of whey protein polymers. *Food Engineering and Physical Properties 65*(1): 139–143.

Moro, A, Báez, GD, Ballerini, GA, Busti, PA and Delorenzi, NJ. 2013. Emulsifying and foaming properties of β-lactoglobulin modified by heat treatment. *Food Research International 51*(1): 1–7.

Morr, C V. 1979. Functionality of whey protein products. *New Zealand Journal of Dairy Science and Technology*.

Mullally, MM, Callaghan, DMO, Fitzgerald, RJ, Donnelly, WJ and Dalton, JP. 1994. Proteolytic and Peptidolytic Activities in Commercial Pancreatic Protease Preparations and Their Relationship to Some Whey Protein Hydrolysate Characteristics 2973–2981.

Mulvihill, DM and Donovan, M. 1987. Whey proteins and their thermal denaturation - a review. *Irish Journal of Food Science and Technology 11*(1): 43–75.

Mulvihill, DM and Ennis, MP. 2003. Functional Milk Proteins: Production and Utilization. In P. F. Fox & P. L. H. McSweeney (eds.), *Advanced Dairy Chemistry: Vol. 1. Proteins* (3rd ed.). New York: Kluwer Academic/Plenum Publishers.

Murray, TK and Baker, BE. 1952. Studies on protein hydrolysis. I.—preliminary observations on the taste of enzymic protein-hydrolysates. *Journal of the Science of Food and Agriculture 3*(10): 470–475.

Mutilangi, W a. M, Panyam, D and Kilara, a. 1996. Functional Properties of Hydrolysates from Proteolysis of Heat-denatured Whey Protein Isolate. *Journal of Food Science 61*(2): 270–275.

Nakamura, T, Sado, H, Syukunobe, Y and Hirota, T. 1993. Antigenicity of whey protein hydrolysates prepared by combination of two proteases. *Milchwissenchaft*.

National Agricultural Statistics Service. 2014. *Dairy Products 2013 Summary*. Washington, DC.

Netto, FM, Desobry, SA and Labuza, TP. 1998. Effect of water content on the glass transition, caking, and stickiness of protein hydrolysates. *International Journal of Food Properties 1*(2): 141–161.

Newport, MJ and Henschel, MJ. 1985. Growth, digestion, and protein metabolism in neonatal pigs given diets containing whey as the predominant or only source of milk protein. *Journal of Pediatric Gastroeneterology and Nutrition 4*: 639–644.

Otte, J, Ju, ZY, Færgemand, M, Lomholt, SB and Qvist, KB. 1996. Protease-Induced Aggregation and Gelation of Whey Proteins. *Journal of Food Science 61*(5): 911–916.

Page, J, Meyer, D, Haines, B, Lagrange, V, Kenney, A and Clark Jr, WS. 2004. *Reference Manual for U.S. Whey and Lactose Products*. Arlington, Virginia.

Patel, HA, Singh, H, Havea, P, Considine, T and Creamer, L. 2005. Pressure-induced unfolding and aggregation of the proteins in whey protein concentrate solutions. *Journal of Agricultural and Food Chemistry* (53): 9590–9601.

Peña-Ramos, EA, Xiong, YL and Arteaga, GE. 2004. Fractionation and characterisation for antioxidant activity of hydrolysed whey protein. *Journal of the Science of Food and Agriculture 84*(14): 1908–1918.

Perea, A, Ugalde, U, Rodriguez, I and Serra, JL. 1993. Preparation and characterization of whey protein hydrolysates: applications in industrial whey bioconversion processes. *Enzyme and Microbial Technology 15*: 418–423.

Pereira, MA, Jacobs, DR, Van Horn, L, Slattery, ML, Kartashov, AI and Ludwig, DS. 2002. Dairy consumption, obesity, and the insulin resistance syndrome in young adults. The CARDIA study. *Journal of the American Medical Association 11*(16): 28–29.

Pouliot, Y, Guy, M-M, Tremblay, M, Gaonac'h, A-C, Chay Pak Ting, BP, Gauthier, SF and Voyer, N. 2009. Isolation and characterization of an aggregating peptide from a tryptic hydrolysate of whey proteins. *Journal of agricultural and food chemistry 57*(9): 3760–4.

Pufulete, M. 2008. Intake of dairy products and risk of colorectal neoplasia. *Nutrition Research Reviews 21*: 56–67.

Rabiey, L and Britten, M. 2009. Effect of protein composition on the rheological properties acid-induced whey protein gels. *Food Hydrocolloids 23*: 973–979.

Rao, Q, Fisher, MC, Guo, M and Labuza, TP. 2013. Storage stability of a commercial hen egg yolk powder in dry and intermediate-moisture food matrices. *Journal of Agricultural and Food Chemistry 61*(36): 8676–86.

Rao, Q, Kamdar, AK and Labuza, TP. 2013. Storage Stability of Food Protein Hydrolysates-a Review. *Critical reviews in food science and nutrition* (August): 37–41.

Rao, Q and Labuza, TP. 2012. Effect of moisture content on selected physicochemical properties of two commercial hen egg white powders. *Food Chemistry 132*(1): 373–384.

Rao, Q, Rocca-Smith, JR, Schoenfuss, TC and Labuza, TP. 2012. Accelerated shelf-life testing of quality loss for a commercial hydrolysed hen egg white powder. *Food chemistry 135*(2): 464–72.

Resmini, P, Pellegrino, L and Batelli, G. 1990. Acurate quantification of furosine in milks and dairy products by a direct HPLC method. *Italian Journal of Food Science 2*: 173–183.

Saint-Sauveur, D, Gauthier, SF, Boutin, Y and Montoni, A. 2008. Immunomodulating properties of a whey protein isolate, its enzymatic digest and peptide fractions. *International Dairy Journal 18*: 260–270.

Sava, N, Van der Plancken, I, Claeys, W and Hendrickx, M. 2005. The kinetics of heat-induced structural changes of beta-lactoglobulin. *Journal of dairy science 88*(5): 1646–53.

Schägger, H and von Jagow, G. 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Analytical Biochemistry 166*(2): 368–79.

Schnickels, RA, Warmbier, HC and Labuza, TP. 1976. Effect of protein substitution on nonenzymatic browning in an intermediate moisture food system *24*(5).

Singh, a. M and Dalgleish, DG. 1998. The Emulsifying Properties of Hydrolyzates of Whey Proteins. *Journal of Dairy Science 81*(4): 918–924.

Singh, H and Havea, P. 2003. Thermal Denaturation, Aggregation, and Gelation of Whey Proteins. In P. F. Fox & P. L. H. McSweeney (eds.), *Advanced Dairy Chemistry: Vol. 1. Proteins* (3rd ed.). New York: Kluwer Academic/Plenum Publishers.

Sinha, R, Radha, C, Prakash, J and Kaul, P. 2007. Whey protein hydrolysate: Functional properties, nutritional quality and utilization in beverage formulation. *Food Chemistry 101*(4): 1484–1491.

Slade, L, Levine, H and Reid, DS. 1991. Beyond water activity: recent advances based on an alternative approach to the assessment of food quality and safety. *Critical Reviews in Food Science & Nutrition 30*(2): 115–360.

Spellman, D, Kenny, P, O'Cuinn, G and FitzGerald, RJ. 2005. Aggregation properties of whey protein hydrolysates generated with Bacillus licheniformis proteinase activities. *Journal of agricultural and food chemistry 53*(4): 1258–65.

Spotti, MJ, Perduca, MJ, Piagentini, A, Santiago, LG, Rubiolo, AC and Carrara, CR. 2013. Gel mechanical properties of milk whey protein–dextran conjugates obtained by Maillard reaction. *Food Hydrocolloids 31*(1): 26–32.

Stamp, JA and Labuza, TP. 1983. Kinetics of the Maillard reaction between aspartame and glucose in solution at high temperatures. *Journal of Food Science 48*(2): 543–544.

Tang, CH, Li, L and Yang, XQ. 2006. Influence of transglutaminase-induced cross-linking on in vitro digestibility of soy protein isolate. *Journal of Food Biochemistry 30*: 718–731.

Taoukis, PS, El, MA and Labuza, TP. 1988. Moisture Transfer and Shelf Life of Packaged Foods. In J. H. Hotchkiss (ed.), *Food and Packaging Interactions*. American Chemical Society.

The NPD Group. 2014. *Protein Perceptions and Needs*. Port Washington, NY.

Ting, BPCP, Gauthier, SF and Pouliot, Y. 2007. Fraction of β-lactoglobulin tryptic peptides using spiral wound nanofiltration membranes. *Separation Science and Technology 42*: 2419–2433.

Toledo, E, Delgado-Rodríguez, M, Estruch, R, Salas-Salvadó, J, Corella, D, Gomez-Gracia, E, … Martínez-González, M a. 2009. Low-fat dairy products and blood pressure: follow-up of 2290 older persons at high cardiovascular risk participating in the PREDIMED study. *The British Journal of Nutrition 101*: 59–67.

Tunick, MH. 2008. Whey Protein Production and Utilization: A Brief History. In C. Onwulata & P. J. Huth (eds.), *Whey Processing, Functionality and Health Benefits*. Blackwell Publishing.

Turgeon, SL, Gauthier, SF and Paquin, P. 1991. Interfacial and emulsifying properties of whey peptide fractions obtained with a two-step ultrafiltration process. *Journal of Agricultural and Food Chemistry 39*(4): 673–676.

UBIC Consulting. 2014. *Protein Hydrolysate Ingredient Market*. Newport Beach, CA.

Unterhaslberger, G, Schmitt, C, Sanchez, C, Appolonia-Nouzille, C and Raemy, A. 2006. Heat denaturation and aggregation of β-lactoglobulin enriched WPI in the presence of arginine HCl, NaCl and guanidinium HCl at pH 4.0 and 7.0. *Food Hydrocolloids 20*(7): 1006–1019.

Van der Ven, C, Gruppen, H, de Bont, DB a. and Voragen, AGJ. 2001. Emulsion Properties of Casein and Whey Protein Hydrolysates and the Relation with Other Hydrolysate Characteristics. *Journal of Agricultural and Food Chemistry 49*(10): 5005–5012.

Van Meijl, LEC. 2010. The role of dairy products and constituents in metabolic risk management 1–121.

Van Meijl, LEC and Mensink, RP. 2011. Low-fat dairy consumption reduces systolic blood pressure, but does not improve other metabolic risk parameters in overweight and obese subjects. *Nutrition, Metabolism and Cardiovascular Diseases 21*(5): 355–361.

Van Meijl, LEC, Vrolix, R and Mensink, RP. 2008. Dairy product consumption and the metabolic syndrome. *Nutrition Research Reviews 21*: 148–157.

Veldhorst, M, Smeets, A, Soenen, S, Hochstenbach-Waelen, A, Hursel, R, Diepvens, K, … Westerterp-Plantenga, M. 2008. Protein-induced satiety: effects and mechanisms of different proteins. *Physiology & behavior 94*(2): 300–7.

Visschers, RW and de Jongh, HHJ. 2005. Disulphide bond formation in food protein aggregation and gelation. *Biotechnology Advances 23*(1): 75–80.

Voutsinas, LP, Cheung, E and Nakai, S. 1983. Relationships of hydrophobicity to emulsifying properties of heat denatured proteins. *Journal of Food Science 48*: 26–32.

Walstra, P, Wouters, JTM and Geurts, TJ. 2006. *Dairy Science and Technology* (2nd ed.). Boca Raton, FL, USA: Taylor & Francis Group/CRC Press.

Wang, Q, He, L, Labuza, TP and Ismail, B. 2013. Structural characterisation of partially glycosylated whey protein as influenced by pH and heat using surface-enhanced Raman spectroscopy. *Food chemistry 139*(1-4): 313–9.

Wang, Q and Ismail, B. 2012. Effect of Maillard-induced glycosylation on the nutritional quality, solubility, thermal stability and molecular configuration of whey protein. *International Dairy Journal 25*(2): 112–122.

Warmbier, HC, Schnickels, RA and Labuza, TP. 1976. Nonenzymatic Browning Kinetics in an Intermediate Moisture Model System: Effect of Glucose to Lysine Ratio. *Journal of Food Science 41*: 981–983.

Warren, RM and Labuza, TP. 1977. Comparison of chemically measured available lysine with relative nutritive value measured by a Tetrahymena bioassay during early stages of nonenzymatic browning. *Journal of Food Science 42*: 429–431.

Weiss, WF, Young, TM and Roberts, CJ. 2009. Principles, approaches, and challenges for predicting protein aggregation rates and shelf life. *Journal of Pharmaceutical Sciences 98*(4): 1246–1277.

Wolfrom, ML, Kashimura, N and Horton, D. 1974. Factors affecting the Maillard browning reaction between sugars and amino acids. *Journal of Agricultural and Food Chemistry 22*: 791–795.

Yudkoff, M, Daikhin, Y, Nissim, I, Horyn, O, Luhovyy, B, Lazarow, A and Nissim, I. 2005. 4th Amino Acid Assessment Workshop Brain Amino Acid Requirements and Toxicity : The Example of Leucine 1 , 2.

Zamora, R and Hidalgo, FJ. 2005. Coordinate contribution of lipid oxidation and Maillard reaction to the nonenzymatic food browning. *Critical Reviews in Food Science and Nutrition 45*(1): 49–59.

Zhou, P, Guo, M, Liu, D, Liu, X and Labuza, TP. 2013. Maillard-reaction-induced modification and aggregation of proteins and hardening of texture in protein bar model systems. *Journal of Food Science 78*(3): C437–44.

Zhou, P and Labuza, TP. 2007. Effect of Water Content on Glass Transition and Protein Aggregation of Whey Protein Powders During Short-Term Storage. *Food Biophysics* (2): 108–116.

Zhou, P, Liu, D, Chen, X, Chen, Y and Labuza, TP. 2014. Stability of whey protein hydrolysate powders: Effects of relative humidity and temperature. *Food Chemistry 150*: 457–62.

Zhou, P, Liu, X and Labuza, TP. 2008a. Moisture-induced aggregation of whey proteins in a protein/buffer model system. *Journal of Agricultural and Food Chemistry 56*(6): 2048–54.

Zhou, P, Liu, X and Labuza, TP. 2008b. Effects of moisture-induced whey protein aggregation on protein conformation, the state of water molecules, and the microstructure and texture of high-protein-containing matrix. *Journal of Agricultural and Food Chemistry 56*(12): 4534–40.

Zhu, D, Damodaran, S and Lucey, JA. 2008. Formation of whey protein isolate (WPI)-dextran conjugates in aqueous solutions. *Journal of Agricultural and Food Chemistry 56*(16): 7113–8.

Zhu, D and Labuza, TP. 2010. Effect of cysteine on lowering protein aggregation and subsequent hardening of whey protein isolate (WPI) protein bars in WPI/buffer model systems. *Journal of Agricultural and Food Chemistry 58*(13): 7970–9.

Appendix A. Browning of WPH Incubated with and without Dextran

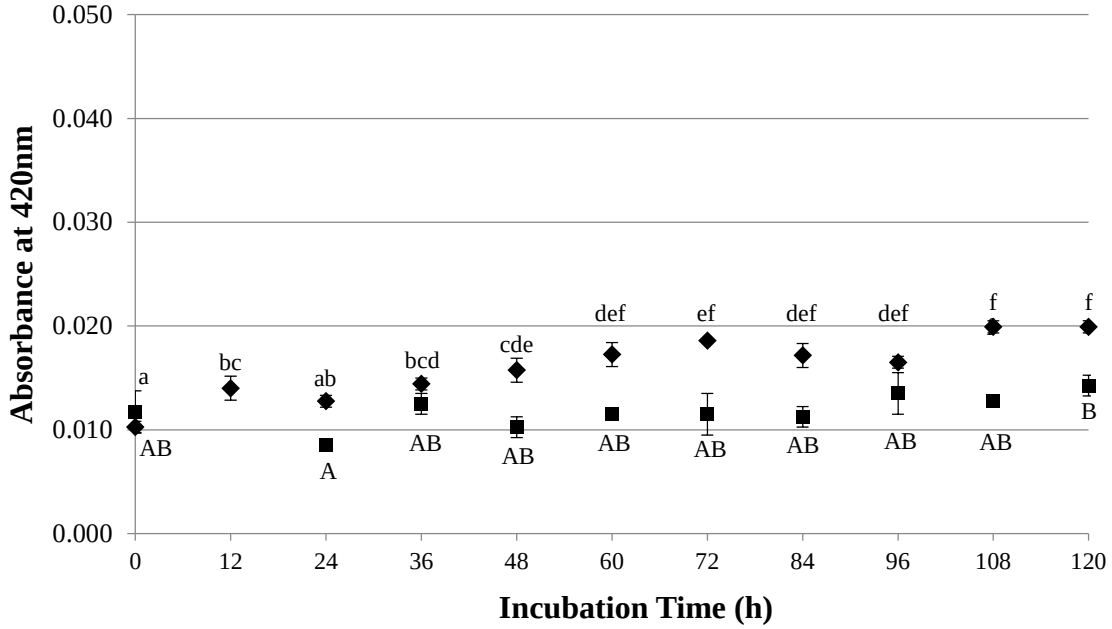

Figure 15. Browning as determined by UV-Visible difference spectroscopy at 420 nm for whey protein hydrolysate (WPH) incubated with dextran (◆) and control WPH incubated without dextran (■) at 60°C for 0-120 h at 49% RH. Error bars represent standard errors (n=3). Different letters above or below the shapes indicate significant differences between different time points according to the Tukey-Kramer multiple means comparison test ($P \leq 0.05$).

Appendix B. Formation of Glycoproteins in WPH Incubated with and without Dextran

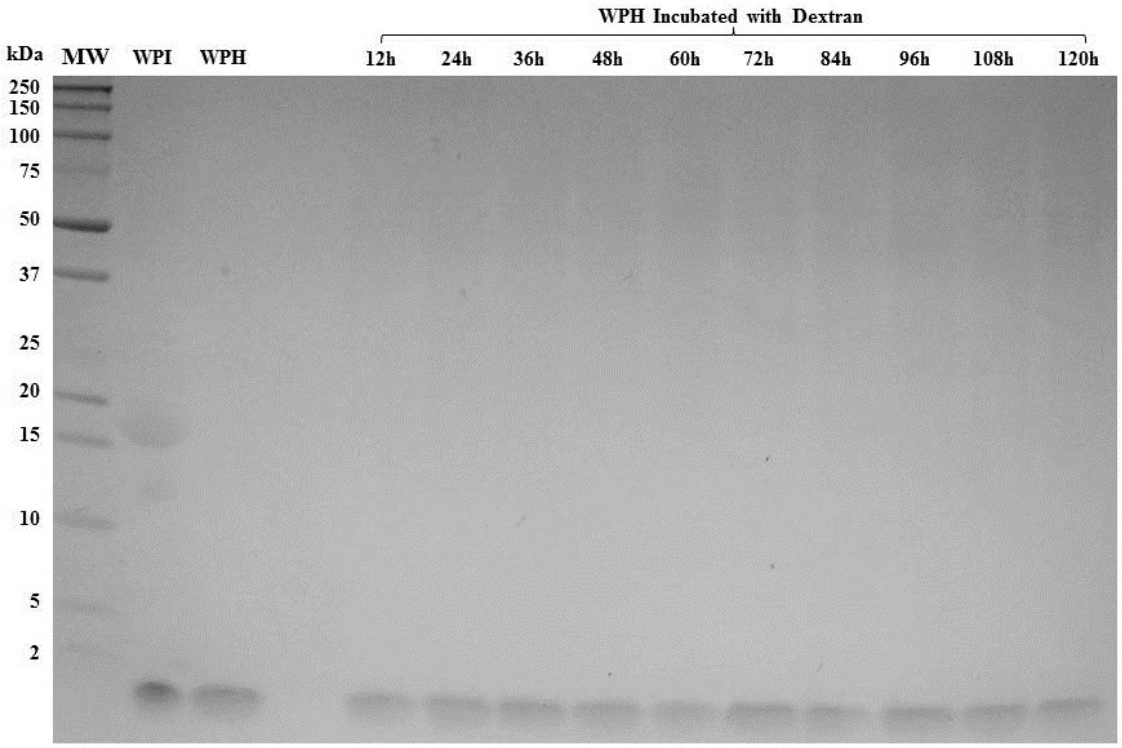

Figure 16. Formation of glycoproteins as visualized by Glycoprotein stained SDS-PAGE for WPH incubated with dextran at 60°C for 0-120 h at 49% RH. MW: molecular weight in kDa. Whey protein isolate (WPI) was run as a reference.

Appendix C. Digestibility of PGWPH and WPH

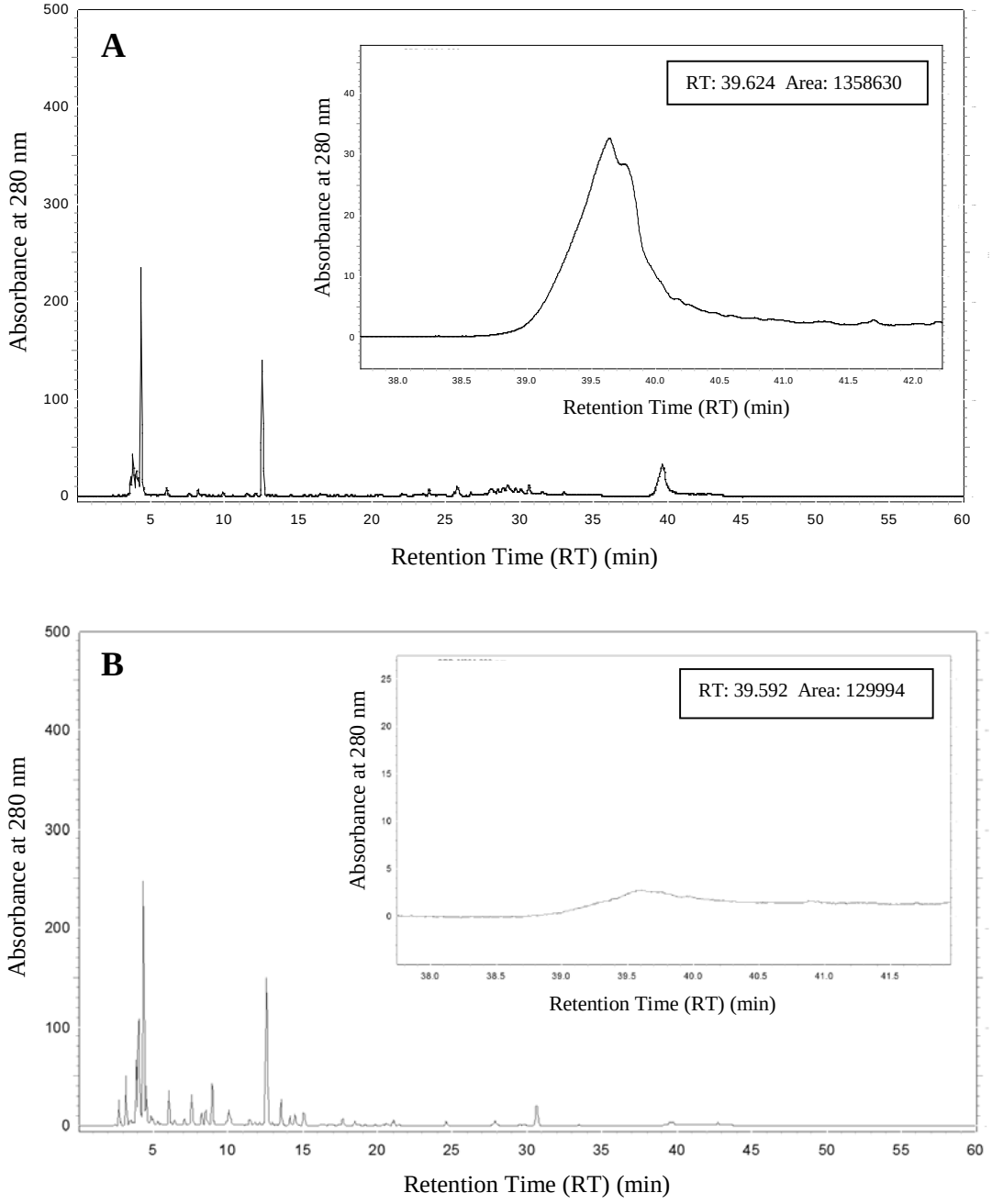

Figure 17. Chromatograms of WPH before (A) and after (B) *in-vitro* digestion with pepsin and trypsin. Chromatogram shows absorbance at 280 nm, scaled to 500 absorbance units. Additional chromatograms indicate selected peak areas used for determination of digestibility.

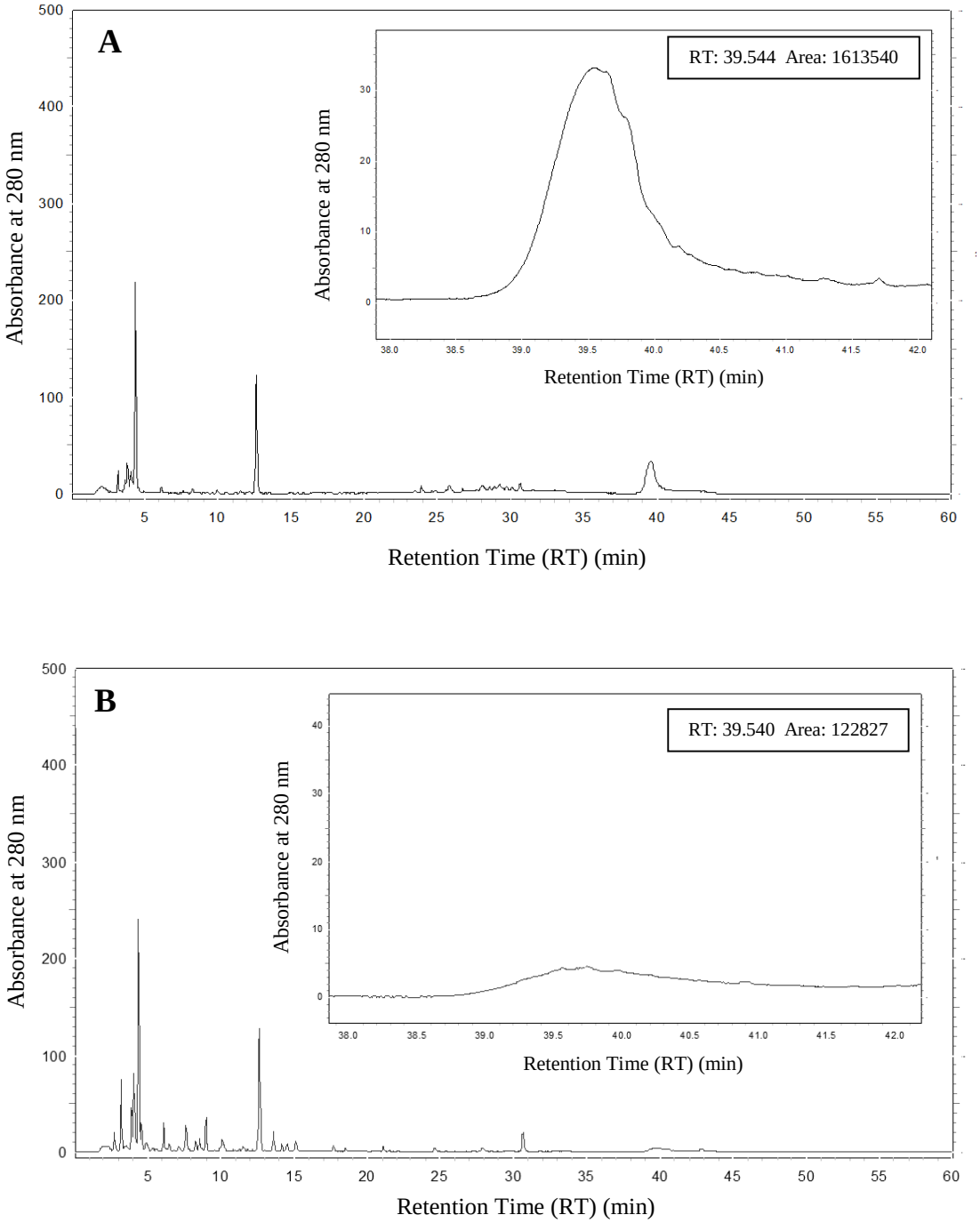

Figure 18. Chromatograms of PGWPH before (A) and after (B) *in-vitro* digestion with pepsin and trypsin. Chromatogram shows absorbance at 280 nm, scaled to 500 absorbance units. Additional chromatograms indicate selected peak areas used for determination of digestibility.

Appendix D – Change in a* and b* values of PGWPH and WPH Stored at 45°C and 31%/65% RH

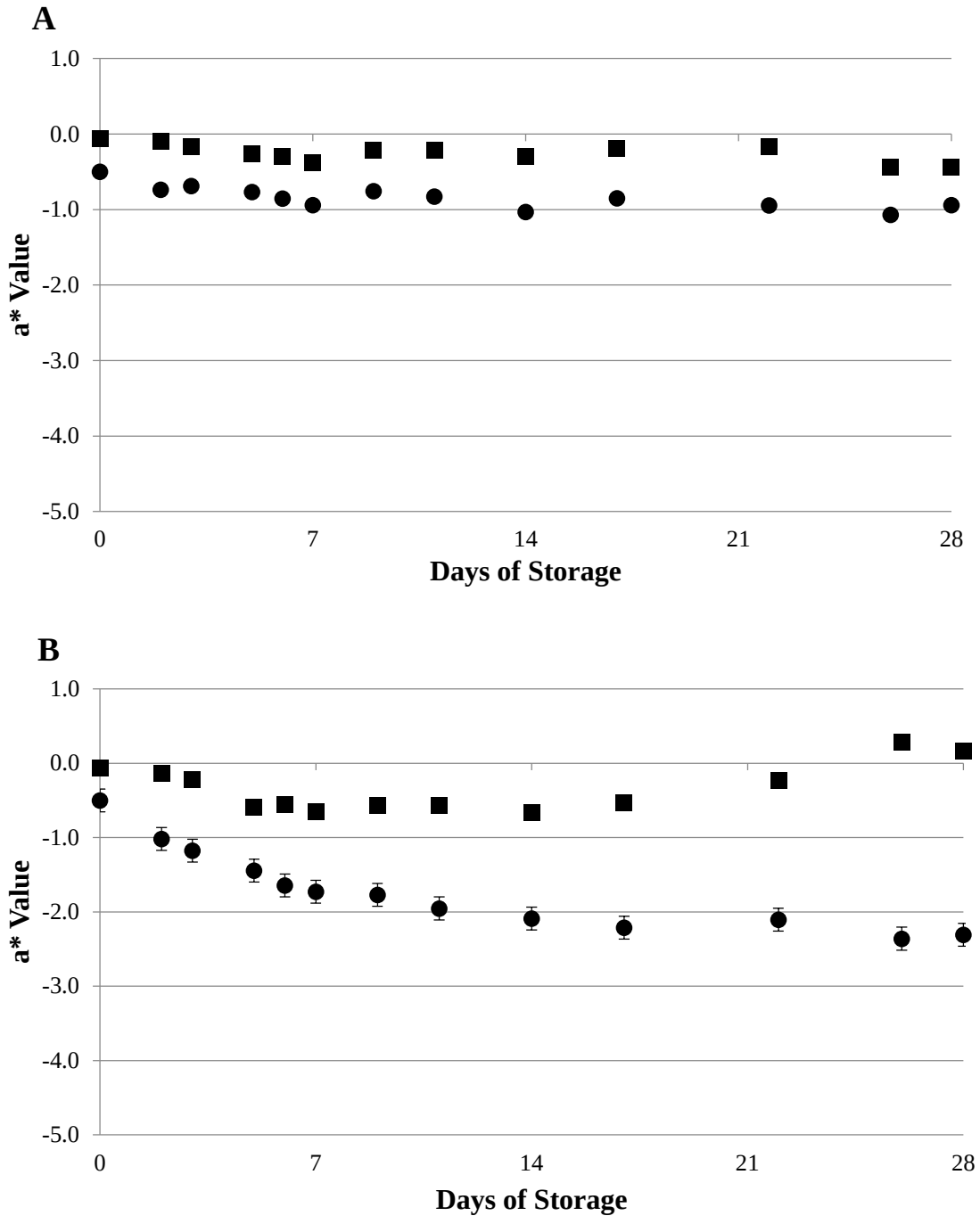

Figure 19. Changes in a* values of PGWPH (■) and WPH (●) during storage at 45°C and 31% (A) and 65% (B) RH. Error bars represent standard error (n=3).

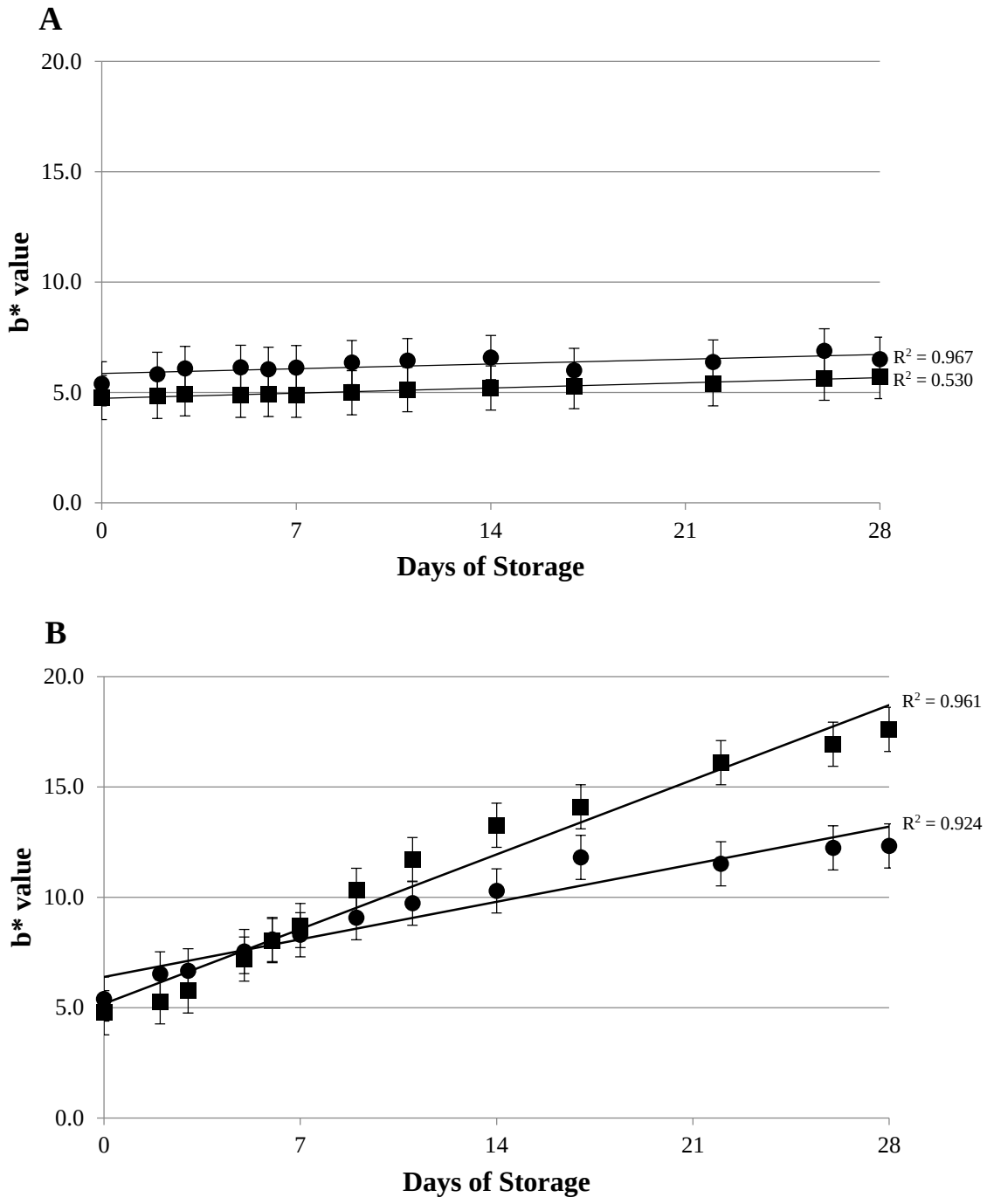

Figure 20. Changes in b* values of PGWPH (■) and WPH (●) during storage at 45°C and 31% (A) and 65% (B) RH. Error bars represent standard error (n=3). R^2 values indicate goodness of fit for each trend-line.

Table 8. Kinetic analysis of zero-order model for the change in *b value as a function of sample type and storage RH at 45°C.

Apparent Linear Model	31% RH		65% RH	
	PGWPH	WPH	PGWPH	WPH
Best fit values				
b^*_{28}	5.7	6.7	18.7	13.1
k x 10^2 (day^{-1})	0.034	0.024	0.48	0.23
95% CI				
b^*_{28}	5.6-5.8[a*]	6.4-6.9[b]	17.5-19.8[d]	12.2-13.9[c]
k x 10^2 (day^{-1})	0.029-0.038[a]	0.01-0.04[a]	0.41-0.54[c]	0.18-0.28[b]
Goodness of fit				
R^2	0.967	0.530	0.961	0.924

b^*_{28}: model predicted values for ΔE after 28 days of storage
k x 10^2 (day^{-1}): reaction rate constant
[*] Values in the same row with different letters are significantly different ($P \leq 0.05$) by the use of a 95% confidence interval (CI).

Appendix E – Change in Peptide Profile of PGWPH and WPH Stored at 45°C and 31% RH

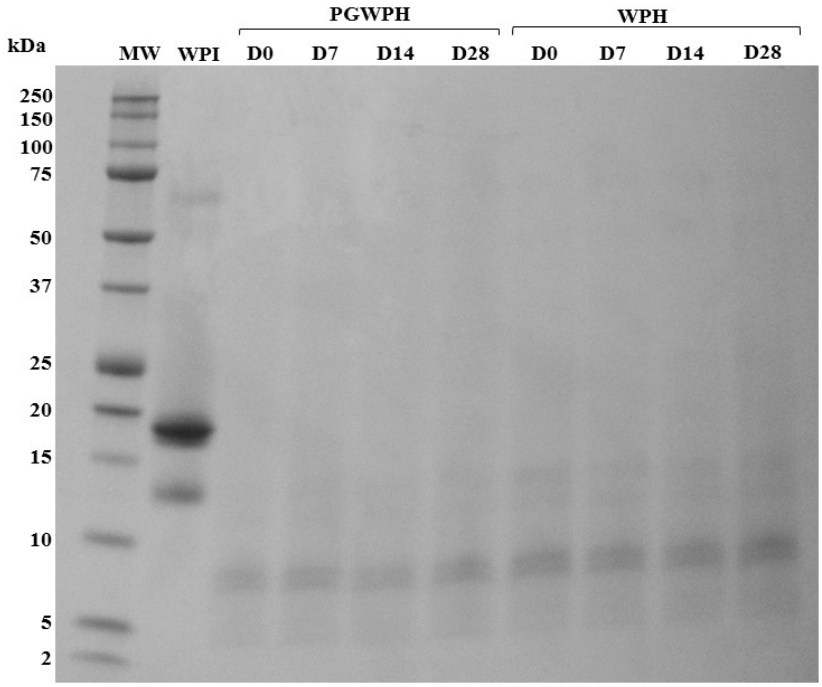

Figure 21. Change in protein/peptide molecular size as determined by Coomassie blue stained SDS-PAGE for PGWPH and WPH stored at 45°C and 31% RH for 7 (D7), 14 (D14), and 28 (D28) days along with non-incubated controls (D0). MW: molecular weight in kDa. Whey protein isolate (WPI) was run as a reference.

Appendix F. Analysis of Variance (ANOVA) Tables for Determining Significant Effects of Treatments

Table 9. Analysis of variance on the effect of incubation time on 304 nm absorbance of WPH incubated with or without dextran.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
WPH incubated with dextran at 49% RH, 45°C	Incubation Time	10	0.017	316.988	0.000
	Error	51	0.000		
WPH incubated without dextran at 49% RH, 45°C	Incubation Time	9	0.001	27.196	0.000
	Error	10	0.000		

Table 10. Analysis of variance on the effect of incubation time on 420 nm absorbance of WPH incubated with or without dextran.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
WPH incubated with dextran at 49% RH, 45°C	Incubation Time	10	0.000	21.413	0.000
	Error	51	0.000		
WPH incubated without dextran at 49% RH, 45°C	Incubation Time	9	0.001	2.724	0.067
	Error	10	0.000		

Table 11. Analysis of variance on the effect of incubation time on % fluorescent intensity of WPH incubated with or without dextran.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
WPH incubated with dextran at 49% RH, 45°C	Incubation Time	9	51274.032	110.480	0.000
	Error	20	464.103		
WPH incubated without dextran at 49% RH, 45°C	Incubation Time	9	11438.132	51.509	0.000
	Error	20	222.062		

Table 12. Analysis of variance on the effect of incubation time on % free amino group loss of WPH incubated with dextran.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
WPH incubated with dextran at 49% RH, 45°C	Incubation Time	9	51274.032	110.480	0.000
	Error	20	464.103		

Table 13. Analysis of variance on the effect of sample type on furosine content of WPH and PGWPH

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
PGWPH, WPH	Sample Type	1	12.611	101.861	0.010
	Error	2	0.124		

Table 14. Analysis of variance on the effect of sample type on % digestibility of WPH and PGWPH

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
PGWPH, WPH	Sample Type	1	5.100	0.488	0.557
	Error	2	10.456		

Table 15. Analysis of variance on the effect of sample type and %RH on L* value after 28 days of storage at 45°C.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
PGWPH, WPH after 28 days storage at 31%/65% RH and 45°C	Sample Type	3	7.115	46.6	0.00
	Error	8	0.153		

Table 16. Analysis of variance on the effect of storage time on L* value of PGWPH stored at 31% RH and 45°C.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
PGWPH stored at 31% RH and 45°C	Storage Time	1	0.317	1.079	0.358
	Error	4	0.294		

Table 17. Analysis of variance on the effect of storage time on L* value of WPH stored at 31% RH and 45°C.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
WPH stored at 31% RH and 45°C	Storage Time	1	1.500	2.765	0.172
	Error	4	0.543		

Table 18. Analysis of variance on the effect of storage time on L* value of PGWPH stored at 65% RH and 45°C.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
PGWPH stored at 31% RH and 45°C	Storage Time	1	22.195	84.758	0.001
	Error	4	0.262		

Table 19. Analysis of variance on the effect of storage time on L* value of WPH stored at 65% RH and 45°C.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
WPH stored at 65% RH and 45°C	Storage Time	1	0.522	0.869	0.404
	Error	4	0.601		

Table 20. Analysis of variance on the effect of storage time on % remaining free amino groups of PGWPH stored at 31% RH and 45°C.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
PGWPH stored at 31% RH and 45°C	Storage Time	7	1.261	6.653	0.001
	Error	16	0.190		

Table 21. Analysis of variance on the effect of storage time on % remaining free amino groups of WPH stored at 31% RH and 45°C.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
WPH stored at 31% RH and 45°C	Storage Time	7	3.268	6.369	0.001
	Error	16	0.513		

Table 22. Analysis of variance on the effect of storage time on % remaining free amino groups of PGWPH stored at 65% RH and 45°C.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
PGWPH stored at 65% RH and 45°C	Storage Time	7	1.167	4.907	0.004
	Error	16	0.238		

Table 23. Analysis of variance on the effect of storage time on % remaining free amino groups of WPH stored at 31% RH and 45°C.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
WPH stored at 31% RH and 45°C	Storage Time	7	2.735	2.213	0.098
	Error	16	1.236		

Table 24. Analysis of variance on the effect of storage time on the surface hydrophobicity index (S_0) of PGWPH stored at 31% RH and 45°C.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
PGWPH stored at 31% RH and 45°C	Storage Time	3	6016.3	0.241	0.865
	Error	8	24976		

Table 25. Analysis of variance on the effect of storage time on the surface hydrophobicity index (S_0) of WPH stored at 31% RH and 45°C.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
WPH stored at 31% RH and 45°C	Storage Time	3	34526	2.272	0.157
	Error	8	25193		

Table 26. Analysis of variance on the effect of storage time on the surface hydrophobicity index (S_0) of PGWPH stored at 65% RH and 45°C.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
PGWPH stored at 65% RH and 45°C	Storage Time	3	60177	2.062	0.184
	Error	8	29179		

Table 27. Analysis of variance on the effect of storage time on the surface hydrophobicity index (S_0) of WPH stored at 31% RH and 45°C.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
WPH stored at 31% RH and 45°C	Storage Time	3	57249	3.526	0.068
	Error	8	16237		

Table 28. Analysis of variance on the effect of storage % RH on the surface hydrophobicity index (S_0) of PGWPH stored at 45°C for 7 days.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
PGWPH stored at 45°C for 7 days	Storage % RH	1	28981	2.591	0.183
	Error	4	11187		

Table 29. Analysis of variance on the effect of storage % RH on the surface hydrophobicity index (S_0) of WPH stored at 45°C for 7 days.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
WPH stored at 45°C for 7 days	Storage % RH	1	251371	16.875	0.015
	Error	4	14896		

Table 30. Analysis of variance on the effect of storage % RH on the surface hydrophobicity index (S_0) of PGWPH stored at 45°C for 14 days.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
PGWPH stored at 45°C for 14 days	Storage % RH	1	136866	2.769	0.171
	Error	4	49419		

Table 31. Analysis of variance on the effect of storage % RH on the surface hydrophobicity index (S_0) of WPH stored at 45°C for 14 days.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
WPH stored at 45°C for 14 days	Storage % RH	1	4406	0.164	0.707
	Error	4	26944		

Table 32. Analysis of variance on the effect of storage % RH on the surface hydrophobicity index (S_0) of PGWPH stored at 45°C for 28 days.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
PGWPH stored at 45°C for 28 days	Storage % RH	1	74103	2.035	0.227
	Error	4	36414		

Table 33. Analysis of variance on the effect of storage % RH on the surface hydrophobicity index (S_0) of WPH stored at 45°C for 28 days.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
WPH stored at 45°C for 28 days	Storage % RH	1	5865.6	0.320	0.602
	Error	4	18333		

Table 34. Analysis of variance on the effect of storage % RH on the water solubility of PGWPH stored at 45°C for 28 days.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
PGWPH stored at 45°C for 28 days	Storage % RH	1	37.216	1.159	0.342
	Error	4	32.119		

Table 35. Analysis of variance on the effect of storage % RH on the water solubility of WPH stored at 45°C for 28 days.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
WPH stored at 45°C for 28 days	Storage % RH	1	0.001	0.000	0.986
	Error	4	2.936		

Table 36. Analysis of variance on the effect of storage time on water solubility of PGWPH stored at 31% RH and 45°C.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
PGWPH stored at 31% RH and 45°C	Storage Time	1	7.848	0.263	0.635
	Error	4	29.869		

Table 37. Analysis of variance on the effect of storage time on water solubility of WPH stored at 31% RH and 45°C.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
WPH stored at 31% RH and 45°C	Storage Time	1	59.265	0.852	0.408
	Error	4	69.557		

Table 38. Analysis of variance on the effect of storage time on the water solubility of PGWPH stored at 65% RH and 45°C.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
PGWPH stored at 65% RH and 45°C	Storage Time	1	10.884	3.107	0.153
	Error	4	3.503		

Table 39. Analysis of variance on the effect of storage time on the water solubility of WPH stored at 31% RH and 45°C.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
WPH stored at 31% RH and 45°C	Storage Time	1	0.149	0.030	0.874
	Error	4	4.980		

Table 40. Analysis of variance on the effect of storage % RH on the pH 3.4 solubility of PGWPH stored at 45°C for 28 days.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
PGWPH stored at 45°C for 28 days	Storage % RH	1	4.268	0.692	0.493
	Error	2	6.168		

Table 41. Analysis of variance on the effect of storage % RH on the pH 3.4 solubility of WPH stored at 45°C for 28 days.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
WPH stored at 45°C for 28 days	Storage % RH	1	141.3	2.149	0.280
	Error	2	65.78		

Table 42. Analysis of variance on the effect of storage time on the pH 3.4 solubility of PGWPH stored at 31% RH and 45°C.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
PGWPH stored at 31% RH and 45°C	Storage Time	1	0.241	0.028	0.883
	Error	2	8.694		

Table 43. Analysis of variance on the effect of storage time on the pH 3.4 solubility of WPH stored at 31% RH and 45°C.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
WPH stored at 31% RH and 45°C	Storage Time	1	104.63	1.568	0.337
	Error	2	66.740		

Table 44. Analysis of variance on the effect of storage time on the pH 3.4 solubility of PGWPH stored at 65% RH and 45°C.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
PGWPH stored at 65% RH and 45°C	Storage Time	1	6.540	1.194	0.389
	Error	2	5.479		

Table 45. Analysis of variance on the effect of storage time on the pH 3.4 solubility of WPH stored at 31% RH and 45°C.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
WPH stored at 31% RH and 45°C	Storage Time	1	2.754	0.266	0.657
	Error	1	10.338		