The Impact of Moisture-Induced Aggregation of Soy Protein Isolate and Hydrolysate During Storage on Product and Nutritional Quality

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Dedication

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Abstract

With the increased interest in protein-based foods in the United States, soy protein isolate (SPI) and hydrolysate (SPH) have become widely used in the food industry. However, during processing and distribution proteins/peptides tend to aggregate when introduced to increased temperatures and relative humidity. When aggregation occurs in a dry protein powder, there is a decrease in processability, product quality, and consumer acceptability. It is therefore necessary to characterize the covalent and non-covalent interactions involved in moisture-induced aggregation as well as their reaction kinetics to aid in the development of technologies to limit aggregation.

The objectives of this study were twofold: (1) to characterize the physicochemical changes of moisture-induced protein/peptide aggregation in SPI and SPH powders during storage as a function of water activity (a_w) and temperature and (2) to determine the nutritional and physiological changes of SPI and SPH during storage when subjected to various a_w and temperatures.

The effects of a_w and temperature during the storage of SPI, SPH, and a 50:50 mixture on several physicochemical characteristics were investigated. Three temperatures (25, 35, and 45°C) and 8 a_w (ranging from 0.05 to 0.79) were employed for the storage study. Prior to storage, intrinsic properties as well as moisture sorption isotherms were determined for each protein powder. Over time, the color change was assessed in a non-destructive manner; also, samples were collected at predetermined time points to monitor the aggregation and resulting changes. Change in color, % solubility, protein/peptide profile, loss of free amino groups, formation of fluorescent Maillard compounds, and denaturation were monitored using well-established methods of analysis. Reaction kinetics were used when possible to better understand the effects of storage parameters and sample types, while also allowing for better predictions at other storage parameters. Lastly, changes in *in vitro* digestibility and angiotensin-converting enzyme (ACE) inhibitory activity were assessed to determine protein aggregation's effects on nutritional and physiological changes.

Storage at a_w above the monolayer moisture value of the soy protein ingredients resulted in the formation of insoluble aggregates. Hydrophobic interactions, disulfide bonds, and covalent linkages induced by the Maillard reaction lead to the observed aggregation; these interactions were most pronounced at 45°C and a_w>0.59. Even though there was a minimal amount of reducing sugars in the protein powders, the development of fluorescent compounds, change in color, and loss of free amino groups indicated that the Maillard reaction occurred. Soy protein hydrolysate was the most susceptible to the Maillard reaction, but SPI and 50/50 had larger decreases in % solubility over time at a_w>0.33 and 45°C. The aggregates formed during storage, however, did not have a significant effect on the % digestibility and ACE inhibitory activity of soy proteins/peptides. These findings can be used to help predict changes in food matrices with similar a_w during storage for optimal utilization of soy protein ingredients.

Table of Contents

List of Tables

Table Page

List of Figures

Figure Page

1. Literature Review

1.1 Introduction and Objectives

Soybeans have been utilized as a rich source of protein and oil for centuries; they are highly abundant worldwide and have an advantage over many animal-based products due to their comparably low cost. However, only in the past few decades has there been a significant surge of soy protein-based products in the United States. Popularity of soy protein, specifically in Western culture, has grown since 1999 when the Food and Drug Administration (FDA) released the health claim that "25 grams of soy protein per day, as part of a diet low in saturated fat and cholesterol, may reduce the risk of heart disease." Since then, research has shown that soy protein – and peptides derived from that protein – provide countless more benefits than were noted only a few years earlier.

With increasing consumer awareness of and demand for healthy foods with functional properties, soy protein is positioned at the forefront. Soy protein provides a good quality and highly digestible protein alternative to those who are allergic to, or prefer not to eat, egg, meat, and/or milk proteins. Soy protein isolate (SPI), which is composed of over 90% protein, provides a vegetable-based source of protein that has excellent functionality in various food products. As a food ingredient, SPI is used to enhance gelation, foaming, water-holding capabilities, and emulsification, just to name a few.

Soy protein hydrolysate (SPH), made from SPI through enzymatic hydrolysis, has enhanced functionalities compared to SPI and is also a source of bioactive peptides. In general, bioactive peptides can be released from proteins *in vivo* by gut enzymes, which randomly hydrolyze peptide bonds, or *in vitro* by controlled fermentation or hydrolysis. The peptides released by gut enzymes can widely vary depending on individual gut microbiota, and may not always be bioactive. By performing *in vitro* enzymatic hydrolysis, the process can be controlled to consistently produce bioactive peptides that can then be directly absorbed in the human gastrointestinal tract.

Soy protein isolate and SPH are widely used as ingredients in ready-to-eat foods when in the form of dry powders. However, during distribution and storage in warehouses for extended periods of time, moisture and temperature abuse may occur causing quality

and nutritional degradation. Research on food proteins has shown that extended and/or aggravated storage leads to a large change in color, a decrease in free amino groups, and aggregation. The aggregation is often due to intermolecular hydrophobic interactions and disulfide bonding. However, the extent of aggregation and the types of bonds/interactions formed is highly dependent on the type of protein; therefore, the changes that occur in egg, casein, whey, and soy protein may differ considerably.

Aggregation can lessen the nutritional quality, reduce consumer acceptability, and make further food processing increasingly challenging. The extent of aggregation depends on the conformation and bonding properties of the protein as well as extrinsic factors such as pH, percent moisture, ionic strength, and protein concentration. A large promoter of aggregation is thermal processing, which causes denaturation and thus conformational change of the proteins. Also, enzymatic hydrolysis can drastically alter the size and conformation of proteins, which may promote aggregation. The surrounding food system also has an impact on the development of aggregation. For example, a food matrix high in reducing sugars would be a good medium for the Maillard reaction, which may lead to protein aggregation. It is necessary to understand how the extent of aggregation leads to change in functional, nutritional, and processing aspects of soy proteins.

With the increase in demand for healthy foods and escalating consumer awareness, functional and bioactive protein ingredients are gaining prominence in the food industry. However, it is unknown how the functionality of soy protein ingredients change over time when subjected to the common stressors of temperature and moisture. In order to effectively use SPI and SPH as food ingredients—maintaining their nutritional, physiological, and overall quality—the trends and rates of their physicochemical, nutritional, and physiological changes must be established. Therefore, the objectives of this work are:

(1) Characterize the physicochemical changes of moisture-induced protein/peptide aggregation in SPI and SPH powders during storage as a function of water activity and temperature.

(2) Determine the nutritional and physiological changes of SPI and SPH during storage when subjected to various water activities and temperatures.

1.2. Significance of Soybeans

1.2.1. Origin and Historical Growth of Soy

Originating in East Asia, the domestic soybean [Glycine max (L.) Merrill] has been cultivated for human use since the eleventh century B.C. (Ho, 1969). There, soybeans have been consumed in traditional forms such as edamame (green, fresh soybeans), nimame (cooked whole soybeans), soymilk, tofu, tofu-yo (fermented tofu), soy sauce, miso, and tempeh. The Japanese introduced Europeans to soybeans in the 1700s; however, the United States did not begin growing the crop until the early 1800s and then only as a forage and pasture crop (Morse, 1950). By the 1920s, the United States began processing soybeans for their oil, leaving the soybean meal as a byproduct fed to cows or used as fertilizer (Smith & Circle, 1978). For the rest of the 1900s, the United States modernized and improved the manufacturing process of traditional soy foods and their popularity began to grow.

Currently, the United States is the world's largest producer and exporter of soybeans, planting them primarily in the Midwest region. In 2013, the United States produced 3.29 billion bushels, making it the third largest field crop after corn and peanuts (USDA-NAS, 2014). Out of all of the oilseeds grown in the world, soybeans have the greatest production share at 57% worldwide (USDA-FAS, 2014). The majority of all soybeans produced are crushed to extract the oil; however, the leftover soybean meal is an extremely valuable component because it is very high in protein. In 2013, the total value of the U.S. soybean crop was more than $43 billion (USDA-NAS, 2014). At 67% of the total world protein meal supply product, soy triumphs over other protein meal sources such as fish, rapeseed, and cottonseed (USDA-FAS, 2014). Based on these statistics, soybeans play an important role in the economy; their merit is based on their extremely valuable composition.

Soybeans consist of approximately 40% protein, 35% carbohydrate, 20% fat, and 5% ash. The carbohydrates in soy include mono- and disaccharides, oligosaccharides, and polysaccharides. The three major sugars in soybeans are sucrose and the oligosaccharides raffinose and stachyose (Bainy et al., 2008). Soybeans are rich in two essential fatty acids, linoleic and α-linolenic fatty acids, which have been shown to be highly beneficial in the prevention of arthritis, diabetes, hypertension, coronary artery diseases, and cancer

(Simopoulos, 1999). Although not high in one particular vitamin or mineral, soybeans are good sources of vitamin C, B vitamins, iron, and calcium (USDA, 2011). Also, soybeans are the richest source of isoflavones, which are nonnutritive compounds that have physiological benefits (Alekel et al., 2000; Yamamoto et al., 2003). All of these aforementioned nutritional benefits of soy have increased their presence in food.

Although soybean oil was originally the predominant reason for growing soy in the United States, there has been a surge in manufacturing soy food and beverages for the last couple of decades. Soy protein ingredients, which are the main ingredients of many soy-based foods, are predicted to continue leading the plant proteins market for many years; they have sustained a 6.5% compound annual growth rate in recent years, which is almost twice as high as the wheat protein segment (Frost & Sullivan, 2012). Originally starting as a small segment of the processed food market, annual soy food sales have increased to $5.2 billion as of 2011 (Soyfoods Association of North America, 2011). The four largest segments—in order of market share—in the soy food and beverage category include energy bars and gels (20.2%), baby food (18.4%), refrigerated non-dairy beverages (14.7), and frozen meat alternatives (10.0%). Out of these four segments, the energy bars and gels had the largest growth of a 20.6% increase from 2008 to 2010 (Mintel Group, 2011). As of 2012, the supermarket sales of meat alternatives have grown by 8% since 2010 to a total of $553 million (Mintel Group, 2013). The large growth of meat alternatives is actually not due to an increase in people adopting a vegetarian diet. Only 7% of all consumers identify as vegetarians while 36% indicate that they use meat alternatives (Mintel Group, 2013). Consumers tend to choose soy-based products mainly because of their desire to switch to a more plant-based diet and their awareness of the health benefits of soy protein-based foods.

1.2.2. Health Benefits of Soy Protein

Although vegetable proteins have often been recognized as inferior sources of protein, soy protein is a good source of essential amino acids. Although soy protein is low in methionine, it is a good source of lysine, which is lacking in most grains (Global Food Forums, 2013). Soy protein has a protein digestibility-corrected amino acid score

(PDCAAS) of 1.00, which is equal to that of egg and milk proteins (Hughes et al., 2011). This makes soy an ideal source of protein for vegans, vegetarians, and those following a more plant-based diet. Along with being a good quality and meat substitute protein, soy protein has been linked to several health benefits. As a consequence of many long-term clinical studies, in 1999 the FDA approved the health claim that "25 grams of soy protein per day, as part of a diet low in saturated fat and cholesterol, may reduce the risk of heart disease" (FDA, 1999). This FDA claim spurred an increase in soy protein food consumption and research of other possible health benefits of soy protein.

As a plant-source of protein, soy has also been highly regarded as an abundant source of phytochemicals, currently estimated at 136 different compounds (Fang et al., 2004). These phytochemicals include oxygenated fatty acids, saponins, and isoflavones. In particular, isoflavones in soybeans have been extensively researched. Isoflavones have gained considerable attention because of their association with the prevention of several chronic diseases including breast cancer development (Yamamoto et al., 2003; Iwasaki et al., 2009), reduction of bone loss in women (Alekel et al., 2000; Somekawa et al., 2001), and decrease in prostate cancer (Ozasa et al., 2004).

Research has shown that hydrolyzing or fermenting soy proteins to produce soy protein hydrolysates (SPH) can give soy protein other properties beyond those just related to intact soy protein. The term, protein hydrolysates, may encompass a mixture of polypeptides, oligopeptides, and amino acids due to the many different ways of hydrolyzing a protein. Breaking down the protein into smaller peptides increases the digestibility of soy protein, which is highly beneficial for infants who have gastrointestinal problems when consuming cow's milk or soy-based formulas. Extensively hydrolyzed proteins, both milk and soy, have also been used as hypoallergenic ingredients resulting in a decrease in gastrointestinal discomfort and allergenicity (Merritt et al., 1990; Serra et al., 2006).

Along with the benefits of increased digestion and reduced allergenicity, hydrolyzing proteins may result in the release of bioactive peptides. Bioactive peptides are specific sequences present within the protein that, when released, positively impact body functions and may ultimately enhance human health (Kitts & Weiler, 2003). Through using

controlled enzymatic digestion, many researchers have been able to produce SPH containing bioactive peptides. Bioactive peptides may have antihypertensive effects by inhibiting the angiotensin-converting enzyme, which is responsible for the production of a vasoconstrictor hormone (Margatan et al., 2013). Additionally, β-conglycinin (a major storage protein in soy) contains many peptide sequences that upon release from the parent protein can reduce inflammation, reduce lipid accumulation, and lower the risk of cardiovascular diseases (Lovati et al., 1996; Manzoni et al., 2003; Martinez-Villaluenga et al., 2009).

1.3. Soybean Protein

1.3.1. Composition

The main storage proteins in soy, glycinin and β-conglycinin, account for approximately 60-80% of the total proteins in soybeans. However, the ratios of glycinin to β-conglycinin can vary greatly due to the many different cultivars of soybeans used to produce soy protein-based ingredients. Glycinin and β-conglycinin are often termed 11S and 7S, respectively, based on their sedimentation coefficients. Other proteins such as γ-conglycinin, lipoxygenase, β-amylases, agglutinins, and the basic 7S globulin are among the remaining proteins (main proteins shown in Table 1). Trypsin inhibitors—mainly the Kunitz and Bowman-Birk—also make up a small portion of the total soy protein composition (Koide & Ikenaka, 1973). The presence of these trypsin inhibitors necessitates the thermal treatment of soy proteins to inactivate them before consumption, as they act as anti-nutritional factors (Friedman & Gumbmann, 1986).

Table 1. Composition and physicochemical characteristics of the major proteins in soy.

Protein	% of soy proteins [ab]	Isoelectric point	Molecular weight (kDa)[ed]	Denaturation temp. (°C) [f]	Sulfhydryl group [gk]	Disulfide group [g]
Glycinin (11S)	36.5-51.0	4.7 [c]	300-380	94.1	12-20[#]	5-13
Acidic polypeptides					6/mol glycinin	
A3 chain			42.0 [e]		4	
A1,2,4 chains			33.6 [e]-37.0		6	
Basic polypeptides			20.7 [e]		6/mol glycinin	
β -conglycinin (7S)	27.8-40.7	4.9-5.0[c]	150-200	76.7	2[#]	0
α'			72.0-82.2 [e]		1	
α			68.0-70.6 [e]		1	
β			48.4 [e]-52.0		0	
γ-conglycinin	5.0-6.2		170[j]			
Basic 7S globulin	3.6	9.1-9.3[i]	168[i]			
Kunitz trypsin inhibitor (2S)	2.9-4.1	3.8 [h]	20.1[h]		4 [h]	2[h]

[a] Murphy & Resureccion (1984). [b] Sato *et al.* (1986). [c] Koshiyama (1972). [d] Fontes *et al.* (1984). [e] Sathe *et al*. (1987). [f] Tang *et al.* (2007). [g] Glycinin data are from Wolf (1993). [h] Koide & Ikenaka (1973) [i] Sato *et al.* (1987). [j] Sato, *et al*. (1984). [k] Utsumi *et al.* (1997). [#] Total sulfhydryl groups.

β-conglycinin, a trimer with a molecular weight of approximately 180 kDa, consists of three subunits held together by hydrophobic bonds (Koshiyama, 1968). The subunits, α, α', and β have molecular weights of approximately 58, 57, and 42 kDa respectively (Petruccelli & Anon, 1995a). All of the subunits' amino acid sequences are similar; however the α and α' subunits contain one cysteine (-SH) residue, while the β subunit does not contain any. Also, the α' subunit has more tryptophan and methionine than the α subunit, while the β subunit does not have any (Utsumi et al., 1997). The trimer displays molecular heterogeneity, as shown in Figure 1, existing as α_3, $\alpha_2\alpha'$, $\alpha_2\beta$, $\alpha\alpha'\beta$, $\alpha\beta_2$, $\alpha'\beta_2$,

and β_3 (Yamauchi et al., 1981). Through examination of the amino acid sequences, researchers determined that β-conglycinin is a glycoprotein with N-linked mannoses at all of its five potential sites (Asn-Xaa-Ser/Thr, where Xaa is any amino acid except Pro): two in the α' subunit, two in the α subunit, and one in the β-subunit (Utsumi et al., 1997; Picariello et al., 2013). The presence of N-glycosylation is important to the structure of β-conglycinin as it affects the protein's folding and stability (Ceriotti et al., 1998).

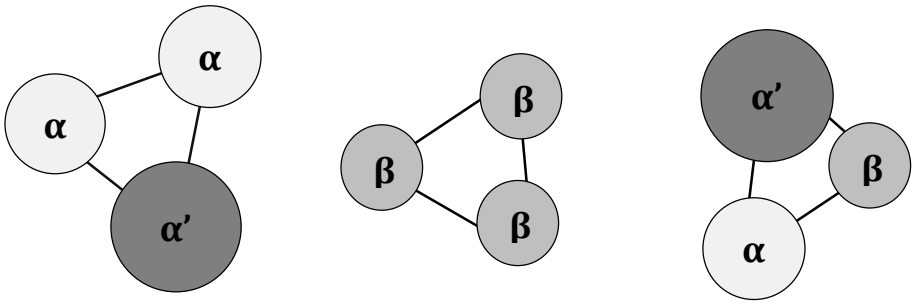

Figure 1. Examples of subunit structures of β-conglycinin.

The larger of the two storage proteins, glycinin, is a hexamer with a molecular weight of approximately 340 kDa (Koshiyama, 1972). The hexamer is made up of two trimers that interact via electrostatic and hydrogen bonding. The three monomers/subunits, which make up each trimer, are bound by hydrophobic interactions (Adachi et al., 2003). Each monomer is made up of two polypeptides, one acidic polypeptide (~35 kDa) and one basic polypeptide (~20 kDa), linked together through one disulfide bond (Staswick et al., 1984). Therefore, there is a total of 12 polypeptides that are arranged into two identical hexagons stacked on top of one another (Figure 2). Five different subunits have been identified and characterized into two groups based on sequence homologies; Group I subunits have more methionine, 2 cysteines, 3 cystines, and slightly lower molecular weights than Group II, which has 2 cysteines and 2 cystines (Utsumi et al., 1997). Through studying the positions of cysteine residues among soybean cultivars, a range of free sulfhydryl residues numbering between 2 to 15 has been determined (Staswick et al., 1984; Wolf, 1993; Utsumi et al., 1997).

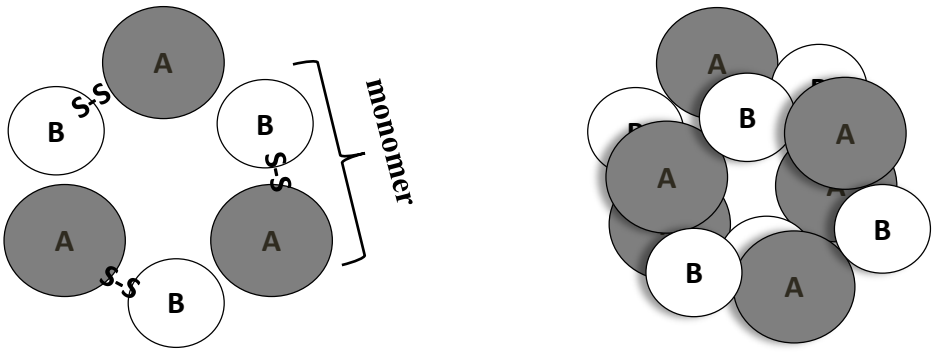

Figure 2. Schematic representation of glycinin (trimer on left, hexamer on right).

1.3.2. Functional Properties

Soy proteins provide an abundance of functional properties when used in food applications: emulsification, viscosity, elasticity, water holding capacity, oil and fat binding, foaming, solubility, and gelation. These properties highly depend on the protein's size, composition, and three-dimensional structure. β-conglycinin and glycinin—and their individual subunits—contribute the most to the functionality of soy protein. The intrinsic factors of soy protein highly define the types of functionalities, while the extrinsic factors influence the extent of functional performance in the food system.

Their relatively large size, amphiphilic characteristic, and structural flexibility give soy proteins favorable interfacial activity (Hettiarachchy & Kalapathy, 1998). Oftentimes, soy proteins are used in ground meat products as well as baked products to maintain and stabilize emulsions. β-conglycinin has greater emulsifying stability and capacity compared to glycinin due to its greater surface hydrophobicity, molecular flexibility, and more open structure (Kinsella, 1979; Wagner & Guéguen, 1999). Due to glycinin's higher amount of disulfide linkages, it cannot unfold as easily as β-conglycinin. For similar reasons, glycinin has less foaming capacity when compared to β-conglycinin (Matsudomi et al., 1985). However, the high amount of disulfide linkages becomes an asset for glycinin when gelation is desired. Glycinin creates a stronger gel at a much faster rate in comparison to β-conglycinin (Kohyama & Nishinari, 1993).

Soy protein functionality can also be influenced by extrinsic factors involved in the processing of complex food systems such as temperature, pH, and ionic strength. The

majority of soy processing involves thermal treatment, which can cause proteins to denature, losing native conformation and possibly affecting their functionality. Due to the presence of many disulfide bonds, glycinin is more thermally stable than β-conglycinin, which is held together mainly through hydrophobic interactions (Utsumi et al., 1997). Through controlling the temperature and time of heating, the extent of denaturation of both major proteins may be controlled to alter functional performance. Depending on the thermal treatment employed, soy protein denaturation can be partially reversible; β-conglycinin will refold and not re-associate but glycinin will only partially refold to its original compact structure (Damodaran, 1988; Iwabuchi et al., 1991). The application of heat, however, can enhance many properties such as water-binding capacity, viscosity, and emulsifying properties (Lopez de Ogara et al., 1992; Nir et al., 1994). For example, heating soy protein isolate at 85°C for 2 minutes gave an emulsion with desirably smaller droplet sizes than non-heated soy protein; this was attributed to the exposure of hydrophobic groups and therefore an increase in surface activity (Nir et al., 1994).

Ionic strength and pH have a large effect on solubility of the protein. Having high solubility is desired as this influences other protein functionalities. To maximize solubility and therefore provide better functionality, it is preferred to keep soy proteins out of their isoelectric point range, which is between pH 4.5-5.0 (Koshiyama, 1972). Ionic strength has an insignificant influence on solubility at a pH near 7.0; but near the isoelectric point, an increase in ionic strength greatly increases solubility (Kinsella, 1979). Conversely, at a pH below the isoelectric point and above 7.0, an increase in ionic strength decreases solubility (Hettiarachchy & Kalapathy, 1998). These effects on solubility are important in complex food systems that require different pH or ionic strength such as acidic beverages or meat product emulsions. To combat the limitations that extrinsic and intrinsic factors often set for soy proteins, researchers have provided many solutions through enzymatic and chemical modifications to produce soy protein ingredients with enhanced functional properties.

1.4. Soy Protein Ingredients

Soy protein ingredients are incorporated into a large variety of products from high-protein beverages and energy bars to bakery products and beef patties. A soy protein ingredient not only enriches a product in protein, but it also enhances functionality and the overall quality of many food products. The emulsification properties of soy protein ingredients are beneficial for applications in meat and bakery products. Soy protein's gelation capabilities, as well as nutritional properties, are highly desired in meat analogues, protein/energy bars, and cereals. As dry powders, soy protein ingredients are utilized mainly in four forms: flour, concentrate, isolate, and hydrolysate.

1.4.1. Soy Flour

Out of all of the soy protein ingredients, soy flour has the lowest protein content at 50-60%. As described by Riaz (2006), food-grade soybeans are made into soy flour by first cleaning the soybeans on sieves using air aspiration to separate out dust, pebbles, and plant tissues. If the flour needs to be enzyme-inactive, the soybeans are steamed under pressure for 20 to 30 minutes and then cooled. Then using grain driers, the soybeans are dried to a moisture level of 10% and tempered to stabilize the moisture content for about three days. Afterwards, soybeans are cracked, dehulled, and rolled into full-fat flakes. To make defatted soy flour, the oil from the flakes is removed using solvent extraction and then the flakes are dried and milled to the desired particle size, leaving 1% or less of oil remaining in the product (National Soybean Research Laboratory, 2010). Defatted soy flour is often used in soups, bakery products, processed meats, and cereals.

1.4.2. Soy Protein Concentrate

Soy protein concentrate (SPC) generally has 65-80% protein and is made from defatted soy flour. Industrially, SPC is produced by treating the flour with moist heat followed by extraction with water, and extraction with aqueous 20-80% ethyl alcohol or acid leaching (Riaz, 2006). These methods remove many of the oligosaccharides present to increase the protein content. After removing the solvent and drying, the SPC is ready for

packaging and shipping. Compared to soy flour, SPC has a blander taste, which is less beany, due to the removal of some flavor constituents. Soy protein concentrate is often used for its emulsification and gelation properties in products such as sausages and ground meats, vegetarian meat analogs, and bakery products (Riaz, 2006).

1.4.3. Soy Protein Isolate

With a protein percent greater than 90%, soy protein isolate (SPI) has the highest amount of protein per gram out of all of the soy protein ingredients. As described by Rhee (1994), SPI is made by solubilizing defatted soy flour in an alkaline solution and brought to a pH of 7.0-8.5. The solution is then centrifuged and the insoluble portion, which contains mostly polysaccharides, is removed. The supernatant is treated with acid to a pH of 4.5 using hydrochloric or phosphoric acid to precipitate the protein into a curd. The curd is washed with water and concentrated by centrifuging; the pellet is re-solubilized and the pH is neutralized to 6.5 to 7.0 followed by spray drying. The resulting product is high in protein while containing lower amounts of flatulence-inducing factors and reducing sugars. Soy protein isolate's high protein content, blander flavor, and increased functionality make it ideal for dairy product replacements, nutritional beverages, protein bars, infant formula, and many other food applications.

1.4.4. Soy Protein Hydrolysate

Soy protein hydrolysate (SPH) is most often made from SPI because it has the highest concentration of protein of all the soy protein ingredients. The production and utilization of SPH are currently increasing due to the associated physiological benefits, the potential reduction of soy protein allergenicity, and the increased digestibility. Along with increased health benefits, hydrolyzing soy protein can enhance many functional properties for increased utilization in a variety of food products.

1.4.4.1. Production

Soy protein hydrolysate is produced by either enzymatic or acidic hydrolysis. Acidic hydrolysis is the cheaper of the two methods, but because the acid can severely degrade

some amino acids (such as tryptophan and cysteine) and have undesired side reactions and products, it is rarely preferred for food ingredient production (Chiang et al., 1999). Enzymatic hydrolysis is a milder process that can be controlled due to the specificity of individual enzymes. Several enzymes including bromelain, pepsin, papain, Alcalase®, and trypsin have been used to produce SPH either by usage individually or as a mixture of several enzymes (Tsumura et al., 2004; Kuipers et al., 2007; Seo et al., 2008). Targeting specific proteins or sites to be hydrolyzed is a type of hydrolysis termed selective hydrolysis. Conversely, limited hydrolysis focuses on obtaining a low degree of hydrolysis (DH), which is the percent of peptides cleaved upon hydrolysis. Both types, selective and limited hydrolysis, may be used to select for various functionalities and/or peptide profiles.

Limited hydrolysis is achieved by controlling the incubation time, temperature, pH and/or enzyme to substrate ratio. It is advantageous to control the DH because many partially hydrolyzed proteins, including soy, have a bitter taste when an excessive amount of low molecular weight hydrophobic peptides are released (Matoba & Hata, 1972). The use of different proteases result in different degrees of hydrolysis where the bitterness becomes a sensory issue. For example, at a 10% DH, SPH produced using Alcalase® has a greater bitter taste than an SPH produced using papain at the same DH (Seo et al., 2008). In general, when maintaining a DH between 2-8%, the hydrolysate can have enhanced functionality, possible bioactivity, and an acceptable flavor. Limited hydrolysis allows for many different types of hydrolysates with a range of improved product uses through utilizing different enzymes and parameters.

Several researchers have focused on selective hydrolysis of either the β-conglycinin or glycinin subunits to examine the direct effect on the protein's functionality and bioactivity. Through using papain and pepsin, Tsumura *et al.* (2004) were able to produce hydrolysates with reduced amounts of β-conglycinin and glycinin, respectively. Through using Alcalase®, Lee *et al.* (1990) demonstrated that β-conglycinin can also be preferentially hydrolyzed over glycinin. By targeting the hydrolysis of a particular soy protein component, ingredients can be tailored to a specific functionality rather than having to rely on only soybean breeding techniques to enhance either β-conglycinin or glycinin. The functional and physiological benefits of selective hydrolysis are further described in

the following sections.

1.4.4.2. Functionality

The main functionality differences between SPH and SPI are a result of the innate differences between a hydrolysate and an intact protein. There is a drastic change in average molecular weights, a change in net charge, and a change in surface hydrophobicity. These changes affect protein/peptide interactions as well as interactions within the system constituents such as water, carbohydrate, and fat, thus ultimately affecting overall functionality.

In the aforementioned study by Tsumura *et al.* (2004), using papain to selectively hydrolyze β-conglycinin and pepsin to hydrolyze glycinin, there were several large differences between the two soy protein hydrolysates produced. With the β-conglycinin hydrolyzed, the protein formed a harder gel, had decreased emulsifying activity, and decreased whippability; the opposite was true when glycinin was hydrolyzed, while leaving β-conglycinin intact (Tsumura et al., 2005). When intact, glycinin forms harder gels than β-conglycinin while β-conglycinin has greater emulsifying activity and foaming capacity. Therefore, these findings prove that enzymatic hydrolysis can enhance desired functionalities inherent to the original protein but not necessarily inherent to each subunit. The ability to tailor the protein ingredient to specific functionalities is important for soybeans because the ratios of β-conglycinin and glycinin range between cultivars, therefore necessitating greater control over subunit functionalities.

Through controlling the DH, the magnitude of the protein's inherent functionalities can be enhanced or diminished. In SPH with hydrolyzed β-conglycinin and/or hydrolyzed glycinin, foaming capacity increased due to the release of smaller molecular weight peptides and the overall increase in surface hydrophobicity (Hettiarachchy & Kalapathy, 1998; Tsumura et al., 2005; Martínez et al., 2009). Having a greater surface hydrophobicity enhances foaming capacity while the small molecular weight peptides can migrate more easily to the interface to stabilize the interface of the foam. The cited studies had a variety of effective DH percentages, ranging from 0.4% to 15%, demonstrating that a small to

moderate DH can enhance functionality. However, some functional properties are difficult to improve through limited hydrolysis, such as solubility. Through hydrolyzing the glycinin subunits to 14% DH, approximately 20% of the solubility was lost at a pH range of 6.0-9.0 (Tsumura et al., 2005). Through using a mixture of Flavourzyme® Type A and Alcalase® 2.4L, Chiang *et al.* (1999) were able to create a SPH with solubility at 100% over a pH range of 2.0-9.0; however, their DH was very large, with all protein molecular weights at or below 4 kDa.

Upon hydrolysis of soy protein under specific conditions, some—but not all—functional properties can be enhanced. By using various enzymes, different hydrolysis conditions (pH, time, temperature, and enzyme to substrate ratio), and by controlling the extent of hydrolysis, researchers can design SPH with desired functionalities for specific applications. Along with increased product functionality, SPH is gaining prominence due to its health benefits from bioactive peptides.

1.4.4.3. Physiological Contributions

Soy protein hydrolysates have several physiological benefits mainly attributed to the release of bioactive peptides. Bioactive peptides are often sequences of 2-9 amino acids that resist further hydrolysis by gut enzymes and can be directly absorbed (Kitts & Weiler, 2003). The release of bioactive peptides is not always guaranteed upon the ingestion of soy protein because the hydrolysis by digestive enzymes is random and non-specific. By completing *in vitro* hydrolysis, as in the production of SPH, the peptides that contribute the most to bioactivity can be selected and released prior to consumption. The bioactive peptides released upon hydrolysis of soy protein contribute to various health benefits such as antihypertensive, cancer-preventative, and antioxidant activity (Hoppe et al., 1997; Park et al., 2007; Margatan et al., 2013). One of the most promising—and most researched—benefits of SPH is the potential antihypertensive activity.

The antihypertensive activity of certain soy peptides is specifically linked to the inhibition of the angiotensin-converting enzyme (ACE). Angiotensin-converting enzyme raises the blood pressure by catalyzing the conversion of angiotensin I to angiotensin II, which is a vasoconstrictor hormone. Additionally, ACE degrades bradykinin, a peptide that

induces vasodilation to lower the blood pressure. Because it has a combination of these two functions, ACE is an ideal enzyme to inhibit when controlling blood pressure. In the United States approximately 77.9 million Americans—or 1 in every 3—have hypertension, which can lead to coronary heart disease, stroke, heart failure, and many other health problems (Go et al., 2013). This makes bioactive peptides with ACE-inhibitory activity all the more important to research.

A significant amount of research has shown that in soy protein, β-conglycinin contains many peptide sequences that can reduce inflammation, reduce lipid accumulation, and lower the risk of cardiovascular diseases (Lovati et al., 1996; Manzoni et al., 2003; Martinez-Villaluenga et al., 2009). When comparing two different SPHs, one with glycinin hydrolyzed and the other with β-conglycinin hydrolyzed, the SPH containing hydrolyzed β-conglycinin subunits had a significantly higher ACE inhibitory activity (Margatan et al., 2013). The use of the enzyme Alcalase® has also been proven to release bioactive peptides from soy protein that have a high degree of ACE activity (Chiang et al., 2006). Alcalase® specifically targets β-conglycinin, making it an ideal enzyme to use for the production of SPH with high ACE-inhibitory activity.

1.5. Moisture-Induced Protein/Peptide Aggregation

Protein/peptide aggregation is defined as the self-association of proteins and/or peptides that can ultimately result in the formation of large polymers, which in turn contribute changes in functionality. In biotechnology and pharmaceutical industries, protein/peptide aggregation can cause serious economic and technical problems, and thus it has been heavily studied within these realms (Liu et al., 1991). However, moisture-induced aggregation is a common phenomenon in food as well; dry food protein powders are especially susceptible to moisture-induced aggregation because of the low initial water activity (a_w) that is needed to maintain the powders' ability to flow. Any increase in moisture in such a dry product can have considerable effects on aggregation. Moisture-induced aggregation can occur as a result of intrinsic factors, thermal denaturation, extrinsic factors, promoters formed during hydrolysis, and the Maillard reaction.

1.5.1. Intrinsic Factors' Effect on Aggregation

The intrinsic factors of a protein such as free sulfhydryl groups, hydrophobic residues, and disulfide groups can greatly influence the extent and type of aggregation. Many researchers have tried to determine the mechanism of aggregation for various proteins, but due to the many factors—intrinsic and extrinsic—involved, each protein seems to have its own unique mechanism. Research on lyophilized proteins such as bovine serum albumin, ovalbumin, and glucose oxidase has shown that intermolecular disulfide bonds, formed via the thiol-disulfide interchange reaction, highly contribute to aggregation (Liu et al., 1991). The presence of free sulfhydryl groups can trigger disulfide interchange, thus causing the formation of aggregates, which in turn result in a large decrease in solubility. Thiol-disulfide interchange has been a driving force in the rapid aggregation of whey and egg protein powders, which contain a relatively high number of free sulfhydryl groups per protein molecule along with many disulfide groups (O'Loughlin et al., 2012; Rao et al., 2013).

Hydrophobic interactions can often be responsible for aggregation as well. The effect of hydrophobic residues on aggregation is often more evident after enzymatic hydrolysis or when thermal treatment results in protein denaturation/unfolding. When proteins unfold, the hydrophobic residues located in the interior become exposed, leading to protein-protein association through hydrophobic interaction as a means to hide from the aqueous surrounding (Damodaran, 2008). This phenomenon is more relevant for globular proteins—such as soy proteins—rather than open-structure proteins. The number of hydrophobic residues and surface hydrophobicity varies between proteins; therefore, the stabilizing effect of hydrophobic interactions also differs depending on the protein. Soy protein association, which often occurs via hydrophobic interactions, can lead to a decrease in solubility and other functional properties where interaction with water is essential, such as gelation and emulsification (Damodaran, 1988; Guo et al., 2012).

1.5.2. Aggregation due to Thermal Treatment and Denaturation

Denaturation of proteins, whether thermal or non-thermal, cause a disruption in the protein's ordered structure and then can subsequently induce aggregation under certain environmental conditions. Heat-induced aggregation often follows thermal denaturation as the polypeptides have disassociated, unfolded, and then re-associated as newly uncoiled polypeptides (Wolf, 1978). The application of heat in processing of food is ubiquitous and therefore must be tightly controlled to achieve desired results. For example, whey protein ingredients are often heated to enhance the gelation properties of β-lactoglobulin (Mudgal et al., 2011). β-lactoglobulin gels can form either fine-stranded or particulate gels. Extensive denaturation forms fine-stranded gels because dimers dissociate to monomers prior to aggregation, while particulate gels mostly retain their dimeric form and only partially unfold during aggregation (Lefèvre & Subirade, 2000). Therefore, the extent of denaturation highly influences the type of aggregates formed.

Denaturation can be a major source of quality loss for protein beverages that undergo a heating step, often pasteurization, due to the loss of solubility. In shelf-stable whey protein beverages, an insignificant amount of aggregation was found to immediately follow heat treatment; however, a significant increase in insoluble aggregates formed over time due to the slower conversion of soluble proteins to aggregates (LaClair & Etzel, 2009). Therefore, to get a full picture of aggregation after denaturation it is necessary to analyze the aggregate formation over time and not simply directly after heat treatment.

Protein denaturation is especially common for soy systems, which are often heated to improve digestibility and enhance some functionalities such as foaming, gelation, and emulsification (Kinsella, 1985; Friedman & Gumbmann, 1986; Lopez de Ogara et al., 1992; Nir et al., 1994). The aggregation patterns of the major soy proteins, glycinin and β-conglycinin, differ when they are heated separately or together. When a 0.5% glycinin solution was heated at 100°C for 4 minutes, approximately 50% of the protein became insoluble with the supernatant containing the acidic subunits and the precipitate containing the basic subunits (Mori et al., 1982). Insolubility of glycinin's basic subunits upon thermal treatment has been reported by several researchers (German et al., 1982; Guo et al., 2012).

However, when a β-conglycinin solution was heated alone, only soluble aggregates were formed and when heated with glycinin, glycinin's solubility significantly increased (Guo et al., 2012). These observations indicated that β-conglycinin might stabilize glycinin during heating; however, the exact mechanism has not been identified. It was hypothesized, however, that a preferential interaction between the basic subunits of glycinin and the β-subunit of β-conglycinin occurred during heating, which prevented the glycinin from precipitating (Utsumi et al., 1984; Petruccelli & Anon, 1995b). Others have hypothesized that the exposed hydrophobic groups and flexible structure of β-conglycinin allow it to surround glycinin and limit the extent of aggregation (Guo et al., 2012).

1.5.3. Extrinsic Factors' Effect on Aggregation

The moisture content, ionic strength, pH, and protein concentration make up the variety of extrinsic factors that influence the onset and rate of aggregation. All of these factors in a food system must be controlled if aggregation is to be avoided. As mentioned previously, higher moisture content lowers the denaturation temperature. Additionally, high moisture content provides greater mobility of proteins, allowing for more protein-protein interactions. This is quite evident in dry protein powders, which when exposed to high humidity start caking, become sticky, and form hard insoluble aggregates (Netto et al., 1998; Zhou & Labuza, 2007); this will be discussed further in latter sections. With more moisture, there is also more of an effect in regards to the other extrinsic factors (ionic strength, pH, and protein concentration) due to greater mobility.

Ionic strength, protein concentration, and pH are interdependent, determining the onset of aggregation based on either shielding or exposure of charges. In general, a high protein concentration enhances the extent and rate of aggregation (Damodaran, 2008). By keeping protein concentrations below 1%, aggregation can be generally avoided (Damodaran & Kinsella, 1982; Kinsella, 1982); however, the concentration of proteins is often above that in food systems touting their richness in protein. An increase of protein concentration from 1% to 4%, using SPI, resulted in the formation of larger aggregates when the pH was above 7.5 as compared to 6.08 and 6.37 pH (Boulet et al., 2000). The

reasoning for this unexpected formation of larger aggregates at neutral pH, rather than the isoelectric point, was because of subparticle expansion due to the increased net negative charge (Boulet et al., 1998). This induced a structural change that exposed positively charged residues, which led to electrostatic links between subparticles. However, the authors noted that this aggregation led to stable, hydrated structures and therefore aggregation may not always lead to precipitation. Most proteins are highly soluble at neutral and alkaline pH 8.0-9.0, but have very minimal solubility at their isoelectric point (generally 4.0-5.0 pH), aggregating due to their lack of electrostatic repulsion (Damodaran, 2008). This aggregation at a pH close to the isoelectric point is highly dependent on protein concentration.

Ionic strength also influences aggregation and is dependent on the pH of the protein system. As mentioned previously, an increase in ionic strength increased soy protein solubility at the isoelectric point but had little to no effect at neutral pH (Kinsella, 1979). Low ionic strength (<0.5 μ) is more influential on proteins with many nonpolar patches—such as soy protein—and will promote protein-protein interaction, resulting in a decrease in solubility (Damodaran, 2008). This decrease in solubility occurs due to the electrostatic instability imparted from the charged surface, which is shielded with higher ionic strength. Conversely, an increase in solubility will occur for proteins with less nonpolar patches, such as β-lactoglobulin. Therefore, the influence of ionic strength and pH are highly dependent on the intrinsic factors of individual proteins.

1.5.4. Aggregation due to Hydrolysis

Sometimes during the enzymatic hydrolysis of a protein, peptides are released that can actually act as promoters of aggregation. During extensive hydrolysis of β-lactoglobulin with *Bacillus licheniformis* protease (BLP), for example, six to seven major peptides have been identified as the key peptides responsible for aggregation and ultimate gelation of whey protein isolate (Otte et al., 1997; Otte et al., 2000). The aggregates, held together by hydrophobic and electrostatic interactions, were mainly composed of a large number of low molecular weight peptides (1.7-4 kDa). In whey protein, a high DH

contributed to higher amounts of aggregation when BLP was utilized (Otte et al., 1996; Creusot et al., 2006). Therefore, certain peptide sequences released from hydrolysis with BLP—or enzymes derived from BLP, such as Alcalse® 2.4L—may lead to protein aggregation.

Interestingly, it was also observed that mixing whey protein peptides (produced through hydrolysis with BLP) with intact protein induced aggregation among non-hydrolyzed proteins (Creusot & Gruppen, 2007b). The aggregates formed through the hydrophobic interaction because BLP broke down many hydrophilic segments, thus preserving the hydrophobic portions (Creusot et al., 2006; Creusot & Gruppen, 2007a). Other researchers have observed similar findings of β-lactoglobulin peptides leading mainly to hydrophobic interactions with the hydrophobic core, promoting a more compact structure (Noiseux et al., 2002). Although aggregation can be disadvantageous in some cases, aggregation in this case was actually desired as it enhanced gelation and showed promise for use as a controlled release in functional food applications, such as delivering bioactive molecules (de Wolf & Brett, 2000).

The hydrolysis of soy protein has also been shown to cause aggregation, although the extent and type of aggregation is highly dependent on the enzyme used as well as the type of substrate. Using subtilisin Carlsberg, chymotrypsin, bromelain, and papain a decrease of solubility of SPH with a DH of 2.2% was found at the neutral pH of 6.5-7.0 (Kuipers et al., 2007). This dependence on the type of enzyme used was unique to SPH in comparison to WPH in the same study. Approximately 40-50% of the cleavage sites for chymotrypsin were located in the hydrophobic regions, while trypsin—which, in contrast to chymotrypsin, did not lead to aggregation in SPH—cleaved only 7-17% of hydrophobic regions. Based on these observations, it is likely that peptides that originate from hydrophobic regions have a tendency to aggregate, specifically those originating from glycinin (Kuipers & Gruppen, 2008). As was observed for the intact β-conglycinin and glycinin, respectively, peptides from β-conglycinin inhibited peptide aggregation while peptides from glycinin had the opposite effect (Tsumura et al., 2005; Kuipers et al., 2006). At a protein concentration of 0.8% and DH of 6.5% (achieved from hydrolysis with subtilisin Carlsberg), glycinin peptides lost approximately 40% of their solubility at a

neutral pH, while the solubility of β-conglycinin and SPI remained the same as the non-hydrolyzed samples (Kuipers et al., 2006). The change in hydrophobicity after hydrolysis, therefore, seems to be a driving force for the aggregation of SPH.

1.5.5. The Maillard Reaction's Effect on Aggregation

In 1912, Louis-Camille Maillard first described the reaction between reducing sugars and amino groups (Maillard, 1912). By 1953, the reaction was explained by Hodge (1953) through a complex series of three stages, which is still relevant today. First, the reducing sugar reversibly reacts with the primary amino group to form a Schiff base, which then may cyclize to form a glycosylamine. Then, the Schiff base undergoes the Amadori rearrangement to form the Amadori product, which is an early intermediate to the whole sequence of reactions. Afterwards, the Amadori compound may degrade or dehydrate into amino or carbonyl intermediates, which can then react with other amino groups to form advanced glycation end products—commonly called AGEs (BeMiller & Huber, 2008). The Maillard reaction is often desired for its resulting flavors and colors, but it can also have a detrimental effect on the quality of food proteins during storage.

The Maillard reaction may result in the formation of protein aggregates through covalent cross-linking. The exact mechanisms for how this aggregation occurs is mostly unknown due to the large number of influencing factors, reactants, and products of the Maillard reaction. As the reaction progresses, the formation of fluorophores, increase in browning, loss of free amino groups, increase in hardness, and reduction of solubility can be indicators of protein aggregation (Labuza & Massaro, 1990; Morales & Boekel, 1997; Matiacevich & Buera, 2006; Rao et al., 2013a; Rao et al., 2013c). These parameters are most often used to measure the extent of the Maillard reaction and any resulting protein aggregation. For example, when inducing the Maillard reaction for ovalbumin by storage with reducing sugars at 55°C and 65% relative humidity (RH), high-molecular weight aggregates formed through covalent linkages between sugars and lysine after just 2 days of incubation (Sun et al., 2004).

The Maillard reaction can be easily induced through the addition of reducing sugars; however, if the reaction is not desired, careful control over formula and storage

parameters as well as elimination of reducing sugars is necessary. Also, reducing sugars vary in their rate of reactivity with the primary amino group to initiate the Maillard reaction; pentoses generally have higher reactivity than hexoses (Laroque et al., 2008). When storing dry egg yolk powder containing less than 0.1% free glucose at 45°C and 0.73 a_w for 28 days, the amount of insoluble aggregates rapidly increased over time (Rao et al., 2013a). Aggregation was found to be the result of covalent intermolecular bonding of proteins induced by the Maillard reaction. Therefore, even if the amount of reducing sugars is practically undetectable, they may still lead to Maillard reaction aggregation during storage.

1.6. Storage Stability Challenges

There are several hurdles to overcome during the distribution and storage of food and food ingredients in order to deliver quality food to the final consumer. Improper storage and handling can lead to food loss during distribution and even at the store and consumers' homes. There is a huge problem of food waste throughout the world; the Food and Agriculture Organization of the United Nations (FAO) estimated that each year approximately a third of all food produced for human consumption is lost or wasted (FAO, 2013). The majority of this food waste occurs during agricultural production, postharvest handling, retail organization, and final consumption (Parfitt et al., 2010). However, approximately 12% is also lost during distribution and storage (FAO, 2013). These losses are dependent on the parameters of distribution and storage such as packaging type, temperature, humidity, and vibrations. Depending on the ingredient or food product, its storage, and its handling, there are many detrimental changes that may affect it prior to reaching the ultimate consumer.

1.6.1. Dry Protein Powders

Protein powders, like most ingredients in the food industry, are manufactured in one place then undergo transportation and storage prior to arriving at the food manufacturing plant where they will be used to produce the final product. During this transportation and

storage, there are many possible adverse conditions that the ingredient can encounter such as pressure changes, increased humidity, and elevated temperatures. To aggravate the protein ingredient even further, there may be additional storage before ultimate usage, whether that be months or years. Although storage studies on dry protein powders are limited, it has been shown that proteins and peptides generally aggregate over time as temperature and a_w levels increase (Liu et al., 1991; Zhou & Labuza, 2007; Rao et al., 2012b). However, it is necessary to characterize the storage effects on the aggregation of different types of proteins, as proteins vary in their physicochemical characteristics.

1.6.1.1. Storage Studies for Egg and Whey Protein Powders

The extrinsic and intrinsic factors dictate the extent and type of aggregation among protein molecules and also between proteins and other compounds in the food matrix, such as carbohydrates. For example, whey protein powder most often contains residual lactose, and egg protein powder contains small amounts of free glucose. As mentioned earlier, reducing sugars such as these in the protein powder matrix will accelerate the Maillard reaction, which may ultimately lead to protein aggregation (Morales et al., 1996; Rao et al., 2012b; Rao et al., 2013a). Even though there was only 0.21% glucose present, the Maillard reaction was found responsible for a large portion of the aggregation of egg yolk powder over time at 45°C and 0.73 a_w (Rao et al., 2013a). Besides the covalent bonding induced by the Maillard reaction, hydrophobic interactions and disulfide bonding also contributed to the overall aggregation.

Even during short-time storage studies, aggregation has been observed in both egg and milk protein powders. After storing whey protein isolate (WPI) and whey protein hydrolysate (WPH) at 45°C and >0.70 a_w for two weeks, WPI did not form insoluble aggregates whereas WPH lost almost half of its' solubility (Zhou & Labuza, 2007). The insoluble WPH aggregates formed through intermolecular disulfide linkages, due to the increased mobility from the addition of moisture. The findings for egg hydrolysates compared to non-hydrolyzed egg protein, was similar. After storage at 23°C for four months, hydrolyzed egg white was significantly harder than dried egg white at a_w 0.54-0.85 (Rao & Labuza, 2012). The researchers noted that the hardening possibly occurred

due to the formation of more liquid bridges in between the many available small peptides, and also due to enhanced hydrophobic interactions. Hydrolysates are often used in the industry as a plasticizer (an ingredient that softens a food product), so it is important to note that this plasticizing effect is dependent on storage factors rather than just the type and amount of peptides in the ingredient.

1.6.1.2. Storage Studies for Soy Protein Powder

Most of the storage studies done on food proteins have focused on the aggregation that occurs over time for egg and whey protein powders, with limited research on soy protein aggregation. Storing soybeans at 57% RH at 20°C for 18 months and 84% RH at 30°C for 8 months, followed with purification of β-conglycinin, resulted in increased surface free sulfhydryl groups, increased disulfide interchange, and decreased surface hydrophobicity as compared to the original non-stored soybeans (Hou & Chang, 2004a). Many researchers have reported very low or no free sulfhydryl groups and disulfide linkages (Thanh & Shibasaki, 1977; Fukushima, 1991). However, others have reported between 0.76 and 2.32 cysteine residues per mole in β-conglycinin's subunits (Koshiyama, 1968; Coates et al., 1985). The high variation of ranges is dependent on soybean cultivar as well as purification technique. During storage, the internal free sulfhydryl groups became exposed while at the same time disulfide interchange also increased resulting in aggregation (Hou & Chang, 2004a).

In the same storage study but with the purification of glycinin, there was a significant increase in surface free sulfhydryl groups, a decrease in internal sulfhydryl groups, and an increase in intramolecular disulfide bonds during the storage at 30°C for 7 months at 84% RH (Hou & Chang, 2004b). These findings, along with a decrease in surface hydrophobicity, indicated structure folding, which led to aggregation via thiol-disulfide interchange. Hou & Chang's work indicates that disulfide bonding and hydrophobic interactions change during storage and thus may lead to aggregation of soy protein powders over time at increased temperatures and RH.

Extended storage of SPI at 45°C and 0.74 a_w resulted in a large decrease in water-soluble proteins when compared to storage at 0.19 and 0.33 a_w (Martins & Netto, 2006).

After equilibrating to 0.74 a_w for 2 weeks at both 25°C and 45°C, there was a complete disappearance of the β subunits of β-conglycinin when observing the protein profile by SDS-PAGE. By 90 days, only the basic subunit of the glycinin protein was detected by SDS-PAGE, which indicated that the other protein subunits were involved in the formation of insoluble aggregates. In both this study and a study by Davies *et al.* (1998) browning was also observed. Browning indicated that the Maillard reaction might play a role in the aggregation process, even though soy protein powders mostly contain non-reducing oligosaccharides and disaccharides. Unlike animal-based proteins, SPI may form Maillard reaction products by interacting with endogenous isoflavones, such as diadzein and genistein (Davies et al., 1998). The decrease in lysine in SPI correlated with the disappearance of genistein and appearance of browning and fluorescing Maillard reaction products.

Because Maillard reaction products can have adverse effects on human health such as an increase in colonic precancerous lesions (Gallaher et al., 1996), further exploration on the nutritional and physiological impact of storage is necessary. Although aggregation has been observed, other studies have focused on the nutritional aspects. After storing SPI and SPH for one year at -18° and 42°C in polyethylene bags (controlled a_w), the *in vitro* protein digestibility via pepsin did not significantly change even though there was a significant decrease in the solubility for both sample types (da Silva Pinto et al., 2005). They hypothesized that this decrease in solubility was due to denaturation, although this was not examined further. Because storage was most likely at a RH not within the optimum for the Maillard reaction, the lack of change in digestion makes sense. However, some Maillard reaction products formed through glucose/glycine reactions in soy proteins, α-lactalbumin, and serum albumin have been shown to inhibit *in vitro* proteolysis by trypsin, erepsin, and pepsin (Adrian & Frangne, 1973). The compounds found to influence this were considered “pre-melanoidin”, low molecular weight Maillard products. The low molecular weight Maillard products inhibited mainly the trypsin activity (O’Brien & Morrissey, 1989). Although there is some evidence of nutritional changes as a result of adverse storage conditions, there is only limited information in regards to soy protein ingredients.

A large gap exists between the storage aggregation research done on intact soy protein and hydrolyzed soy protein. Storage of lyophilized SPH at -20°C for 44 days resulted in aggregation attributed mainly to hydrophobic interactions (Lv et al., 2009). However, it is important to note that high molecular weight aggregates were not readily observed. The aggregation observed through hydrophobic interactions is supported by the previously mentioned studies that observed aggregation due to the release of peptides from hydrophobic regions of the soy proteins (Kuipers et al., 2007; Kuipers & Gruppen, 2008). However, the lack of storage studies on SPH at various temperatures and water activities limits the understanding of the physicochemical changes that may occur during storage. Understanding the physicochemical changes and finding ways to avoid these changes during storage is imperative for utilization of this functional ingredient to its maximum capacity.

1.6.2. Intermediate Moisture Foods

Intermediate moisture foods (IMFs) such as protein/energy bars provide unique challenges due to their water activities ranging between 0.55-0.90 a_w. Within this range, there is increased moisture content and so the proteins have more mobility to interact more readily with one another. When storing a model IMF system using whey protein and phosphate buffer at 45°C for 3 months, a 70% loss of solubility and a decrease in denaturation enthalpy were observed (Zhou et al., 2008). These findings, along with the increased hardening of the protein/buffer model system proved that moisture-induced whey protein aggregation does form over time without the addition of other ingredients (such as reducing sugars). A similar study with a model IMF system using 55% hen egg white protein powder and 45% water, showed a large increase in the formation of fluorescent compounds as well as a 22% loss of solubility (Rao et al., 2012a). The insoluble aggregates formed were determined to be due to intermolecular disulfide linking and Maillard-induced covalent linking of protein molecules.

Along with a high moisture content, IMFs often contain varying amounts of fat and sugar to make the model that much more complex. The addition of reducing sugars will accelerate the Maillard reaction, increasing browning as well as hardening of the protein

bars due to aggregation. In contrast, the addition of fat will have a softening effect, but is often undesirable to consumers looking for healthy protein and energy bars. Finding the right ratio and type of sweeteners and fats is essential for creating a shelf stable IMF. Through the use of polyols such as propylene glycol, sorbitol, and maltitol in the production of WPI bars, a soft texture was maintained due to the low level of insoluble aggregate formation (Liu et al., 2009). The same effect was also demonstrated in egg protein bars; between the use of maltitol or a 50% high-fructose-corn-syrup/50% corn syrup, the use of maltitol resulted in a softer protein bar (Rao et al., 2013a). Therefore, although moisture-induced aggregation may be an inherent problem in protein bars, there are possibilities to lower its deteriorative effects.

1.7. Storage Stability Tests

Completing storage testing for dry protein powders is essential to examine the trends and rates of changes in functionality and nutritional quality over time. After producing the dry powder, the product is packaged and sent to a manufacturing facility. Once packaged, the product has already begun changing and this rate of change is dependent on the environmental stressors it is exposed to such as relative humidity and temperature. To properly assess these stressors and be able to extrapolate what the rates may be at various water activities and temperatures, a variety of factors must be tested. A minimum of three different temperatures and/or three different water activities is necessary for extrapolating changes at other temperatures and/or water activities (Bell, 2007). During and after the storage study has been completed, a variety of tests can be administered to examine how the introduction of varying levels of moisture and temperature affected the protein.

1.7.1. Denaturation and Differential Scanning Calorimetry

The use of differential scanning calorimetry (DSC) on proteins provides very valuable information, including the glass transition temperature (T_g), the denaturation temperature (T_d), and the change in enthalpy (ΔH). The physicochemical changes of proteins over time are closely related to the glass transition temperature (T_g), the

temperature/moisture point below which the product is brittle (glass) and above which the product is soft and chewy (rubber). During storage of proteins with varying relative humidities, the T_g significantly decreases as the a_w increases (Zhou & Labuza, 2007; Rao & Labuza, 2012). This has important implications on aggregation because the change in T_g due to temperature and a_w variation can help predict when protein aggregation may occur. For example, when the storage temperature was 10°C above the T_g at a specific a_w, protein aggregation in WPH occurred (Zhou & Labuza, 2007).

There is a large variety of different DSC instruments that differ in the amount of sample that can be loaded, the range of temperatures used, and the rates of temperature change. By changing these different parameters, the aggregation and gelation of a protein and effects of pre-treatments can be examined. For example, when trying to induce gelation of whey proteins through the addition of 85-100 mM NaCl, researchers were able to detect denaturation of α-lactalbumin and β-lactoglobulin followed by a definite exothermic peak for aggregation (Fitzsimons et al., 2007). However, this finding was only possible due to the large sample size of 850 mg used. Defining aggregation through DSC measurements remains difficult because the most common DSC instruments only allow sample sizes of approximately 20 mg.

1.7.2. Moisture Sorption Isotherms

When characterizing the moisture-induced aggregation of proteins over time, it is important to determine the moisture sorption isotherm, which provides information on how the protein gains and loses moisture depending on the environment's RH. Plotting a moisture sorption isotherm allows for better predictions of a_w or moisture if one or the other is known at the time of sample collection. Also, the isotherm can be used to determine moisture loss or gain when ingredients with known moisture sorption isotherms are added together.

Isotherms for food and food ingredients typically follow one of three different types of isotherms: type I (logarithmic-type curve), type II (sigmoidal curve), and type III (L-shaped curve) (Brunauer, 1945). It is typical for most dry protein powders to have a type II isotherm when plotting the Guggenheim-Anderson-de Boer (GAB) isotherm model

(Labuza & Altunakar, 2007). The type II isotherm is divided up into three different zones, which have been described in detail by Reid & Fennema (2008), to better understand the water absorption as seen in Figure 3.

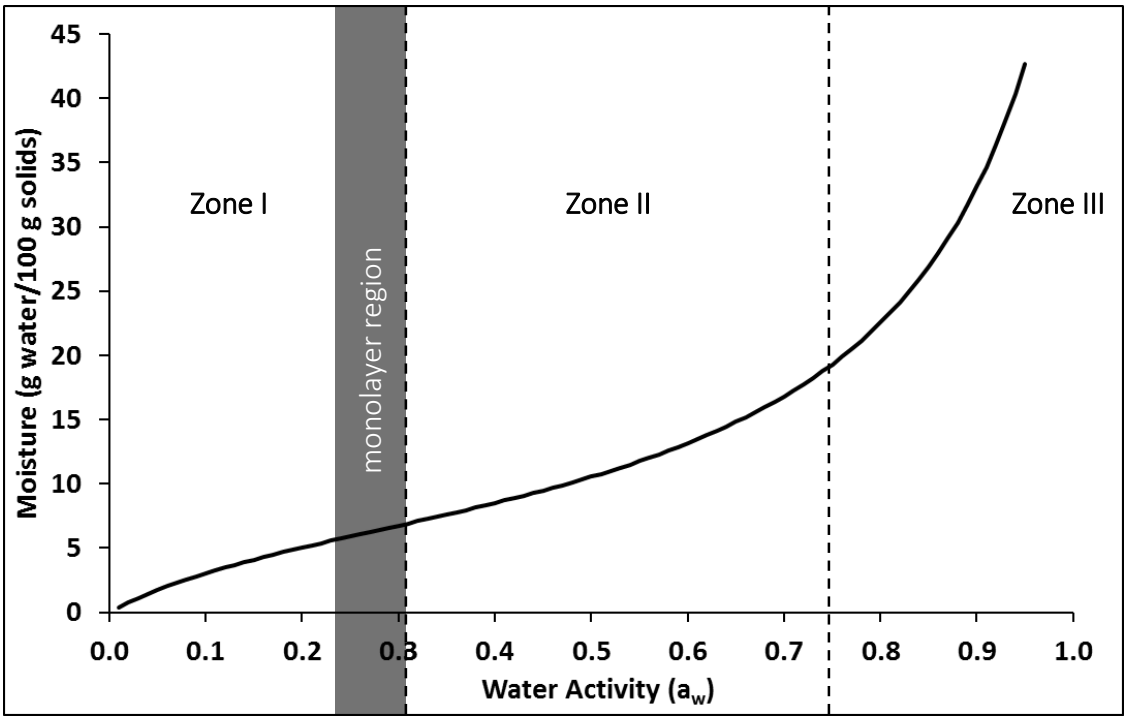

Figure 3. Generalized Type II Moisture Sorption Isotherm at 25°C.

Zone I (a_w < 0.25) represents the water that is the least mobile and is strongly associated with the material. This water is unable to freeze or be removed easily by drying, it behaves as part of the solid (Reid & Fennema, 2008). At the high end of zone I exists the monolayer (m_0), the moisture content at a specific a_w at which the food is at its most stable state. The monolayer tends to lie within the range of 0.2-0.4 a_w for most foods and food ingredients. Zone II (0.30 < a_w <0.75) encompasses where water is adsorbed/absorbed in multilayers at the multiple "sorption sites" represented by the polar or active groups of solute molecules (Reid & Fennema, 2008). Moisture added in the lower end of zone II acts as a plasticizer and lowers the glass transition temperature. An increase in a_w of 0.1 within this range above the m_0 can result in a 2 to 3 times decrease in shelf life (Labuza & Bell,

2000). This type of water is more mobile than the tightly bound m_0 water, but not as much as water absorbed above this point, referred to as bulk or "free" water. Zone III ($a_w > 0.75$) represents bulk water, which can be frozen easily, removed by drying, and also remain available for microbial growth and enzyme activity. Water added at and above a_w 0.75 causes a glass-rubber transition and has a high increase in molecular mobility, which can lead to great increases in rates of deteriorative reactions.

The GAB isotherm model is most widely used because it allows for a good prediction of the moisture sorption isotherm from a_w 0.05 to 0.90 (Bizot, 1983). It also provides three values that help characterize the protein's uptake of moisture: the monolayer moisture value (m_0), the multilayer factor constant (*k*), and the surface heat constant (*C*). The constant *k* is the measure of the difference of free enthalpy of the substance at pure liquid and the layers above the monolayer (Labuza & Bell, 2000). The surface heat constant provides an indication of how the water is bound within the system; for example, a higher *C* correlates to water being more tightly bound (Rao & Labuza, 2012). By understanding the moisture sorption isotherm of protein ingredients, the molecular mobility and rates of deteriorative reactions such as non-enzymatic browning, lipid oxidation, and bacterial growth can be predicted more accurately.

1.7.3. Other Methods to Assess Aggregation

The most obvious change during protein aggregation is a change in molecular weight of the original proteins and peptides present within the system. This has lead many researchers to assess molecular weight changes through sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), size exclusion high-performance liquid chromatography (SE-HPLC), and capillary electrophoresis (CE). In SDS-PAGE, using various buffers such as SDS (used to dissociate non-covalent bonds) and β-mercaptoethanol (used to break disulfide bonds), the types of bonding contributing to aggregation can be elucidated. This provides a qualitative assessment of the aggregation and types of bonds formed, and can also provide some quantitative measurements through the use of densitometry analysis to determine the intensity of protein and peptide bands. The use of SE-HPLC can also provide a quantitative measurement of the molecular weights

of aggregates forming over time and is highly sensitive. The use of CE is not as widespread as SDS-PAGE and SE-HPLC but it provides similar information; with a chromatogram-type readout, it provides quantitative molecular weight measurements.

To examine the types of interactions and bonds formed during aggregation, measurement of solubility in different buffers, at various pHs, and/or at different ionic strengths is often employed. Solubility is highly dependent on the protein concentration, pH, and ionic strength. These parameters, therefore, must be controlled in order to present accurate data. Solubility is commonly determined as the amount of soluble protein in the solution after applying a centrifugal force. Soluble protein content is often determined by total nitrogen percent obtained using common methods such as Dumas on a LECO® Nitrogen Analyzer or Kjeldahl. Through determining the solubility in water, information on how the protein may function during food processing can be obtained. Solubility in water can be related to many functional properties such as gelation and emulsifying capabilities.

1.8. Conclusion

Soy protein provides a host of nutritional, physiological, and functional benefits, with the added advantage that they are relatively low in price when compared to egg, casein, and whey proteins. Therefore, there is great potential for the use of soy proteins in functional foods. However, SPI and SPH are susceptible to aggregation and detrimental changes if they are abused during distribution and storage. Ideally, the protein powders should be stored at refrigerated temperatures and at a relative humidity similar to their monolayer—approximately 5-11% RH. Specifically, moisture-induced aggregation can result in a great loss of functionality due to the loss in solubility and flowing during food processing. There are five main modes that lead to moisture-induced aggregation of proteins: intrinsic factors, protein denaturation, extrinsic factors, aggregation-promoting peptides formed during hydrolysis, and the Maillard reaction. Aggregation has been extensively studied for various proteins in the pharmaceutical world as well as protein-based food systems. However, the majority of the aggregation studies for soy protein have

focused solely on thermally induced aggregation as it relates to gelation and not in a dry system under various storage conditions.

Storage studies done on soy protein are limited and insufficient to draw complete conclusions on how the protein changes over time at various temperatures and a_w conditions. Results from the studies examined determined that aggregates formed during storage may be due to hydrophobic interactions, disulfide interchange, and possibly even the Maillard reaction. However, most of the studies have only examined glycinin and β-conglycinin separately when they are naturally found together. Furthermore, there is scarcely any data on the changes of SPH during storage. Specifically, no work has been done to find out if the bioactivity of SPH changes during storage.

Through exploring the physicochemical, physiological, and nutritional changes in SPI and SPH over time at various water activities and temperatures, their modes of degradation can be determined. Specifically looking at the changes caused by aggregation will help elucidate the mechanisms and the parameters that cause it. It is predicted that hydrophobic interactions, disulfide bonds, and covalent bonds from the Maillard reaction will drive aggregation at high temperatures and water activities. Once knowing the types of aggregation that occur, research can be better tailored to limit this type of aggregation for SPI and SPH.

2. Physicochemical Changes in Soy Protein Isolate and Hydrolysate Powders During Extended Storage

2.1. Overview

During processing and distribution of protein powders, proteins/peptides tend to aggregate when exposed to high relative humidity (RH) and temperatures. When aggregation occurs in a dry protein powder, there is a decrease in processability, product quality, and consumer acceptability. This study characterized the physicochemical changes, including moisture-induced protein/peptide aggregation, in soy protein isolate (SPI), soy protein hydrolysate (SPH), and a 50:50 mixture of SPI and SPH powders during storage as a function of water activity (a_w) and temperature. Several physicochemical changes were monitored during storage including color change, % solubility, protein/peptide profiling, loss of free amino groups, protein denaturation, and production of fluorescent Maillard compounds. Decreased solubility was observed mostly in samples stored at higher a_w. Loss in solubility was attributed to aggregation through hydrophobic interactions, disulfide linkages, and covalent linkages induced by the Maillard reaction. Soy protein isolate experienced the most aggregation, while SPH was the most susceptible to the Maillard reaction. These findings can be used to help predict changes in food matrices with similar a_w during storage for optimal utilization.

2.2. Introduction

Soy protein isolate (SPI) and hydrolysate (SPH) are part of the rapidly growing protein ingredient segment in the global food market. Soy protein leads the global plant protein ingredient section with a value at $5.2 billion as of 2011 and a volume share at approximately 53.4%; this includes soy protein concentrate, SPI, and textured soy protein (Soyfoods Association of North America, 2011; Frost & Sullivan, 2012). Soy protein's popularity is predicted to remain high due to its positioning as a sustainable and affordable protein with a complete nutritional profile. With a sustained compound annual growth of 6.5%, the volume of soy protein ingredients should reach over 1.3 million metric tons by 2018 (Frost & Sullivan, 2012). As research in academia and industry continues to improve soy protein's taste and functionality, the possible substitution of more common whey and egg protein ingredients with soy proteins is becoming a reality. With the increasing use of soy protein isolates and concentrates will also come higher usage of SPH, a key competitor in the functional protein ingredient segment.

In recent years, the usage of protein hydrolysates in functional foods and beverages has increased due to the health benefits associated with their bioactive peptides and reduced allergenicity (Kitts & Weiler, 2003). Bioactive peptides from SPH have been linked to various health benefits including antihypertensive, cancer-preventative, and antioxidant activity (Hoppe et al., 1997; Park et al., 2007; Margatan et al., 2013). Soy protein hydrolysate is used in infant formula, soy sauce, sports nutrition foods/beverages, and enteral formulas. With the ability to enhance SPI's many functionalities, such as foaming, water-holding capacity, gelation, and emulsification upon limited and/or selective hydrolysis, SPH is a flexible and valuable protein ingredient (Hettiarachchy & Kalapathy, 1998; Chiang et al., 1999; Tsumura et al., 2005; Martínez et al., 2009; Bae et al., 2013). However, with the increased usage of SPI and SPH in the food industry also comes the increased probability of their exposure to abusive temperature and relative humidity (RH) during distribution and storage. These adverse conditions can cause detrimental changes to the quality of the protein ingredients.

During processing, distribution, and storage, protein ingredients may be exposed to non-ideal temperatures and RH, which can cause loss of functionality, nutritional quality,

and consumer acceptance. Specifically, moisture-induced aggregation can rapidly occur during dry ingredient storage or within food products that have water activities above the protein's monolayer (m_0) moisture value. Insoluble aggregates can decrease functional properties where interaction with water is essential, such as gelation and emulsification (Damodaran, 1988; Guo et al., 2012). Aggregates formed as a result of the Maillard reaction decrease nutritional quality through a loss in lysine (Labuza & Massaro, 1990; Morales & Boekel, 1997) and reduced digestibility (O'Brien & Morrissey, 1989). Aggregation from the Maillard reaction, hydrophobic interactions, and disulfide bonding also causes hardening of protein bars (Zhou et al., 2008a; Rao et al., 2013a), subsequently decreasing consumer acceptability. It is therefore necessary to characterize the covalent and non-covalent interactions involved in moisture-induced aggregation as well as their reaction kinetics to aid in the development of technologies to limit aggregation.

Hydrolysates tend to form more insoluble aggregates as compared to intact proteins during storage (Netto et al., 1998; Zhou & Labuza, 2007; Rao & Labuza, 2012). The aggregates formed by whey and egg white hydrolysates have been attributed to the development of disulfide bonds, hydrophobic interactions, and covalent bonds induced by the Maillard reaction. Milk and egg proteins have been the focus for the majority of moisture-induced aggregation research (Rao et al., 2012b; Rao et al., 2013b; Zhou et al., 2014), leaving a lack of information on soy proteins and their hydrolysates.

Aggregation of soy protein has been mainly studied directly following thermal treatment, and thus was related to its gelation property rather than its storage stability. Glycinin and β-conglycinin, the major protein components in SPI and SPH, may have opposing roles during aggregate formation. Glycinin and its peptides has been shown to promote aggregation, while β-conglycinin and its peptides inhibited it (Tsumura et al., 2005; Kuipers et al., 2006). Limited research has been done on soy protein hydrolysate aggregation during storage. During storage of SPH at -20°C, peptides less than 10 kDa formed aggregates of approximately 20 kDa, mainly due to hydrophobic interactions (Lv et al., 2009). However, the extent and type of aggregation induced during storage of both SPI and SPH at various a_w have not been characterized.

With the limited number of research findings reported on the storage stability of soy

protein powders, it is necessary to characterize the changes in their overall quality over time to allow for maximum utilization in various food and beverage systems. The main purpose of this study was to characterize the physicochemical changes of moisture-induced protein/peptide aggregation in SPI and SPH powders during storage as a function of water activity and temperature. Through examining the changes in color, % solubility, polymerization, loss of free amino groups, and fluorescence, the degradative behaviors and aggregation can be characterized.

2.3. Materials and Methods

2.3.1. Materials

Defatted, minimally denatured soy flour was generously provided by the Archer Daniels Midland (ADM) Company (Decatur, IL, USA); Alcalase® 2.4L FG was kindly provided by Novozymes North America, Inc. (Franklinton, NC, USA); glucose oxidase/peroxidase (GOPOD) reagent buffer and D-glucose standard solution was purchased from Megazyme (Wicklow, Ireland); bicinchoninic acid (BCA) assay kit was purchased from Thermo Scientific™ Pierce™ (Rockford, IL, USA); standard color plates (yellow, gray, white, pink, blue, and green) were purchased from Hunter Associates Laboratory, Inc. (Fairfax, VA, USA); SDS-PAGE reagents, molecular weight standards, and pre-poured gels were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA); Pronase from *Streptomyces griseus* was purchased from Roche Diagnostics (Indianapolis, IN, USA); reagent grade chemicals were purchased from Fisher Scientific (Pittsburgh, PA, USA) and Sigma-Aldrich (St. Louis, MO, USA).

2.3.2. Preparation of SPI

Minimally heat-treated and defatted soy flour was used to prepare SPI according to the method outlined by Tsumura *et al.* (2004). Soy flour was dissolved in deionized distilled water (DDW) (1:10 w/v); with the pH adjusted to 7.5 using 2M NaOH, the solution was stirred for 1 hour at room temperature. The solution was then centrifuged at 5,000 x g for 30 minutes to separate the insoluble material. The supernatant was retrieved and

adjusted to a pH of 4.5 with 2M HCl to precipitate the protein. Then the solution was centrifuged at 5,000 x g for 10 minutes, at which point the precipitate was collected, redispersed in DDW (1:4 w/v), neutralized to a pH of 7.5, and lyophilized. The lyophilized sample was ground to a smaller particle size using a stainless steel blade coffee grinder (KitchenAid®, St. Joseph, MI, USA), and homogenized with other SPI batches.

2.3.3. Preparation of Soy Protein Hydrolysate

For each replicate of SPH made, a sample of SPI (an amount equivalent to 5.0 g of protein) was dissolved in 98 mL of DDW and adjusted to a pH of 7.5 using 2M NaOH. The SPI solution was heated to 55°C (Alcalase's optimum temperature) for 10 minutes in a water bath with gentle stirring. After the solution reached 55°C, 2 mL of Alcalase solution (0.024 AU-A/mL) solution was added. The solution was then incubated for 45 minutes while continually stirring; for the duration of the incubation, the pH was maintained at a pH of 7.5 by adding 2M NaOH. After incubation, the solution was boiled for 5 minutes, to inactivate the enzyme, and lyophilized. The lyophilized sample was ground to a smaller particle size using a stainless steel blade coffee grinder (KitchenAid®, St. Joseph, MI, USA), and homogenized with other SPH batches.

2.3.4. Storage Study Experimental Design

Soy protein isolate, SPH, and a 50/50 mixture of SPI and SPH (from herein referred to as 50/50) were stored at 3 different temperatures (25, 35, and 45°C) and 8 different water activities (a_w). Desiccators with salt slurries or desiccant were used as the chambers to control the a_w; the a_w for the same salt slurries varied at different temperatures (Table 2). Each sample type (SPI, SPH, 50/50) was stored in separate plastic Decagon cups to be monitored for a_w changes throughout storage. Samples to be checked for color and collected at various time points were stored in a glass Petri dish in a thin, flat layer to allow for maximum water adsorption. Samples to be collected and measured for changes in weight as well as solubility were stored in glass HPLC vials with the same starting mass of 50 mg of protein on a dry basis. Samples were collected at pre-determined time points and subsequently stored at -40°C until analysis.

Table 2. Approximate a_w values of salt slurries and desiccant at different temperatures obtained from direct readings on Aquasorb VSA and Labuza et al. (1985).

Salt Slurry/Desiccant	Temperature (°C)		
	25	35	45
Drierite®	0.05	0.05	0.05
LiCl	0.11	0.11	0.09
$MgCl_2$	0.33	0.32	0.23
K_2CO_3	0.44	0.40	0.33
$Mg(NO_3)_2$	0.54	0.50	0.43
$CoCl_2$	0.64	0.59	0.59
NaCl	0.74	0.75	0.71
KCl	0.84	0.83	0.79

2.3.5. Analysis of Protein Content

To determine the protein content of the dry powder and liquid samples, the AOAC Official Method 968.06 Dumas nitrogen combustion method (AOAC International, 1998) was followed using a Nitrogen Analyzer (LECO® TruSpecN™, St. Joseph, MI, USA). For all soy protein sample types, a nitrogen conversion factor of 6.25 was utilized.

2.3.6. Analysis of Moisture Content

The moisture content for the protein powders was determined Karl Fischer method utilizing the Aquatest CMA Karl Fischer coulometric titrator (Photovolt Instruments, Minneapolis, MN, USA). Samples were prepared in triplicate and methanol blanks were prepared in duplicate in round bottom flasks for extraction of moisture. The amount of sample used each time depended on the presumed moisture content; therefore, if there was more moisture in the sample, less sample quantity was needed (Table 3). Approximately 20g of methanol was added to each sample, the flasks were tightly sealed, and then the flasks were shaken at 100 rpm for 18-20 hours at room temperature. After 18-20 hours of extraction, a syringe was used to remove 1 mL of the methanol extract without disturbing the solids of the sample. A silicon rubber square was used to seal the needle of the syringe and then the syringe, containing the sample, was weighed. The extract was injected into the Karl Fischer coulometric titrator to obtain R (μg) and then the emptied syringe was

weighed to determine the mass of extract injected. The moisture content calculations can be found in Appendix A.

Table 3. Moisture content determination to approximate sample mass needed.

Sample moisture content (%, dry basis)	Sample mass needed
100	≤ 10 mg
50	10 – 20 mg
10	20 – 100 mg
1	100 – 300 mg
0.1	300 – 1000 mg
0.01	1 – 5 g
0.001	5 – 10 g
0.0001	≥ 10 g

2.3.7. Analysis of Total Carbohydrate

Total carbohydrate in the samples was determined following the AOAC Official Method 988.12 phenol-sulfuric acid method (AOAC International, 1988). Dispersions (10 mg solids/mL water) of SPI, SPH, and 50/50 were prepared and analyzed in triplicate. A glucose standard curve utilizing 10, 20, 30, 40, and 50 μg glucose/mL water was constructed to approximate total carbohydrate present in each sample (Figure 23, Appendix B). Samples and standards were prepared and analyzed as outlined by the AOAC method without modification.

2.3.8. Determination of Reducing Sugars

Although a total carbohydrate assay was used, it was necessary to determine if there were any sugars present with reducing activity, which would lead to the Maillard reaction during storage. Several assays were employed to analyze for the monosaccharides, disaccharides, and oligosaccharides present in the samples prior to storage. The methods utilized, dinitrosalicylic acid (DNS) assay, liquid chromatography/mass spectrometry, and a glucose enzymatic assay, are described briefly below.

2.3.8.1. Dinitrosalicylic Acid Assay

The DNS method outlined by Miller (1959) was followed to obtain approximate amounts of reducing sugars present. Dispersions of SPI and SPH (50 mg solids/5 mL DDW) were prepared in triplicate, centrifuged at 15,682 x g for 10 minutes, and the supernatant was removed. An aliquot (400 μL) of the supernatant was mixed 1:1 with DNS reagent (1% dinitrosalicylic acid, 0.2% phenol, and 0.05% sodium sulfite in 1% sodium hydroxide) in a test tube, then tubes were capped and vortexed. The samples were incubated for 5 minutes at 100°C in a water bath, removed to cool, 200 μL of 40% of sodium potassium tartrate (also referred to as Rochelle salt) was added, and the samples were vortexed. A glucose standard curve consisting of 100, 200, 300, 400, 500, and 600 μg glucose/mL DDW (Figure 24, Appendix B) was constructed to calculate concentration (mg/mL) of glucose, which was interpreted as percent reducing sugars, as the assay was not specific for glucose. The samples' and standards' absorbance at 540 nm was read using a DU 640B® UV/Vis spectrophotometer (Beckman Coulter, Inc., Brea, CA, USA).

2.3.8.2. Liquid Chromatography/Mass Spectrometry (LC/MS)

Sugars present in the SPI and SPH samples were extracted with 70% 200 proof ethanol (10:1 ethanol to sample solids) and vortexed for one minute. Samples were stirred for 1 hour at room temperature and then centrifuged at 10,000 x g for 20 minutes. The supernatant was removed and extracted once more with 70% 200 proof ethanol. The excess ethanol was evaporated under nitrogen for 2 hours and the remaining residue was lyophilized. Lyophilized samples were dissolved in DDW to make a 10% solution, and 10 μL was injected into an LC/MS system. A standard solution of stachyose, raffinose, sucrose, and glucose was injected as well to aid in peak designation. Separation was carried out using a LC-10AD system (Shimadzu Corp., Kyoto, Japan), consisting of a LC-10AD dual piston pump, CTO-10A oven, SCL-10A system controller, and Degassit 6324 degasser. The column used was a 7.8 x 300 mm Transgenomic CARBOSep CHO-411 with sodium form ligand-exchange and 20 μm bead size. The oven temperature was set at 70°C, DDW was used as the eluent, and the flow rate was set at 0.3 mL/min. The eluent from the LC column was passed into an electrospray ionization (ESI) interface of Micromass ZQ

mass spectrometer (Waters, Milford, MA, USA). The ionization conditions were: source temperature of 140°C, desolvation temperature of 400°C, cone gas flow of 100 L/h, desolvation gas flow of 600 L/h, capillary voltage 4 kV, cone voltage 75 V, extractor voltage 2 V, RF lens voltage 0.2. Positive ion spectra were monitored using selective ion recording mode and the following ions were monitored: m/z 203, 365, 527, 689, 851, 1013, corresponding to sodium adducts of hexose-derived oligosaccharides with degrees of polymerization of 1-6. Mass Lynx 4.1 software was used.

2.3.8.3. Enzymatic Assay for Glucose Detection

Because glucose is one of the main monosaccharides in soybeans (Bainy et al., 2008; Eldridge et al., 1979), an enzymatic assay specific for glucose was utilized. The samples prepared for the LC/MS were used for the enzymatic assay. Samples, a reagent blank (sodium acetate buffer, 100 mM, pH 4.5), and the D-glucose standards of 10, 20, 30, 40, and 50 μg glucose/mL [made by diluting D-glucose (1.0 mg/mL in 0.2% benzoic acid) in sodium acetate buffer] were incubated with the GOPOD reagent [*p*-hydroxybenzoic acid and sodium azide (0.4% w/v), pH 7.4] in a 1:1 ratio at 40°C for 20 minutes. After incubation, the absorbance was read at 510 nm against the reagent blank. The amount of glucose was determined using a standard curve (Figure 25, Appendix B).

2.3.9. Determination of Water Activity

The water activity (a_w) of the samples was determined using the AquaLab Vapor Sorption Analyzer (Decagon Devices, Pullman, WA, USA). The meter's accuracy was verified using AquaLab verification standards near to the samples' estimated a_w. Samples were placed in plastic a_w cups, ensuring that the level of sample did not exceed half of the cup's height. The samples were read according to the manufacturer's instructions. If the a_w obtained from the meter was in question, duplicate or triplicate readings were taken to verify the correct a_w.

2.3.10. Determination of Degree of Hydrolysis

Each sample of SPH was prepared in triplicate by solubilizing in DDW (1:10 w/v) and centrifuging at 15,682 x g for 10 minutes. The resulting supernatant was mixed with DDW (1:10 dilution) to a final dilution of 1:100 w/v. Then the samples were assayed for protein content using the Thermo Scientific™ Pierce™ BCA assay kit, following the manufacturer's instructions. The degree of hydrolysis (DH) was determined following the o-phthaldialdehyde (OPA) method as outlined by Nielsen *et al.* (2001). Serine (diluted 1:10,000 w/v) was used as the standard to aid in the calculation of the DH. Water mixed with OPA reagent was used as the blank. An aliquot (400µL) of sample was added to 3 mL of OPA reagent, vortexed, and allowed to sit for two minutes before reading the absorption at 340 nm using a DU 640B® UV/Vis spectrophotometer (Beckman Coulter, Inc., Brea, CA, USA). Calculations were carried out using **Equations 1-4**. Example calculation can be found in Appendix C.

Equation 1

$$\text{Serine} - \text{NH}_2 = \frac{\text{OD}_{\text{sample}} - \text{OD}_{\text{blank}}}{\text{OD}_{\text{standard}} - \text{OD}_{\text{blank}}} * 0.9516 \frac{\text{meqv}}{\text{L}} * 0.01 * \frac{100}{\text{X} * \text{P}}$$

Equation 2

$$\text{h} = \frac{(\text{Serine} - \text{NH}_2 - \beta)}{\alpha}$$

Equation 3

$$\text{DH} = \frac{\text{h}}{\text{h}_{\text{total}}} * 100$$

Equation 4

$$\text{Corrected DH} = \text{DH}_{\text{SPH}} - \text{DH}_{\text{SPI}}$$

Where:

Serine – NH_2 = meqv serine NH_2/g protein

OD_{sample} = absorbance of sample at 340 nm

OD_{blank} = absorbance of sample at 340 nm

$OD_{standard}$ = absorbance of sample at 340 nm

X = g sample

P = Protein % in sample

0.1 = sample volume in liters

h = number of hydrolyzed bonds

β = 0.342 (specific for soy protein)

α = 0.970 (specific for soy protein

h_{total} = total number of peptide bonds per protein equivalent (7.8 specific to soy protein)

2.3.11. Moisture Sorption Isotherm Generation

Moisture sorption isotherms for SPI, SPH, and 50/50 were created using AquaLab Vapor Sorption Analyzer (VSA) (Decagon Devices, Inc., Pullman, OR, USA) coupled with the VSA Downloader software. Approximately 500 mg of the sample was weighed into the machine's metal cup. The current moisture content (as determined by Karl Fischer) was input to track the change in weight and thus the change in moisture content as the relative humidity (RH) changed. For creating a dynamic vapor sorption (DVS) isotherm, the test began at 0.05 a_w and stepped up to 0.95 a_w at 0.10 intervals. Only after the weight stabilized for 3 reading points (indicating that the sample a_w had reached equilibrium) was the RH increased to the next highest. For creating the dynamic dewpoint isotherm (DDI), the test also began at 0.05 a_w and ended at 0.95 a_w; the difference from the DVS is that there was a continual increase to the end a_w by going in steps of 0.01 a_w. By setting a lower step level, the resolution of the isotherm was increased.

The completion of one DVS isotherm took approximately 3 days while the DDI took approximately 1 day. Both were run to ensure accurate isotherm modeling. To determine the monolayer (m_0), the surface heat constant (*C*), and the multilayer factor (*k*), the Guggenheim-Andersen-deBoer (GAB) isotherm model was created for SPI, SPH, and 50/50 using the equations found in Appendix D.

2.3.12. Color Analysis by Chroma Meter

The samples' color change over time was assessed using a Chroma Meter CR-221 (Minolta Camera Co., Osaka, Japan). Before analysis of the samples, the meter was

calibrated with the white CR-221 calibration plate (Minolta) and six color standards (yellow, gray, white, pink, blue, and green). The color of the samples was measured following the manufacturer's instruction in the absolute measuring mode, using the CIE (International Commission on Illumination) 1976 $L^*a^*b^*$ color space system. Using a different location in the Petri plate of the sample for each reading, triplicate readings of each sample were taken. The L^* values (lightness) extend between 0 for black and 100 for white; the positive a^* values are red 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.

Samples stored at all temperatures and a_w were tested for color change over time. To measure the overall change in color over time, the total color difference (ΔE) was calculated (**Equation 5**). The ΔE takes into account the differences between the L^*, a^*, and b^* values from one time point to the next. Example calculation can be found in Appendix E.

Equation 5

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

Where:

ΔE = total color difference

ΔL^* = change in L^* value from day 1 to day of measurement

Δa^* = change in a^* value from day 1 to day of measurement

Δb^* = change in b^* value from day 1 to day of measurement

2.3.13. Loss of Free Amino Groups

The loss of free amino groups was assessed by the OPA method as outlined by Vigo *et al.* (1992), with modifications. Samples (10 mg/mL), prepared in triplicate, were dissolved in 1% sodium dodecyl sulfate (SDS) to enhance solubility of the majority of soy proteins through partial unfolding. After thoroughly vortexing the samples, they were centrifuged at 15,682 x g for 10 minutes and the supernatant was removed for analysis.

Samples were diluted 4x for the OPA method and subsequently diluted 2x more for a total of 8x dilution from the original solution for protein analysis using the Thermo Scientific™ Pierce™ BCA assay kit, following the manufacturer's instructions. The OPA reagent was made by dissolving 80 mg of OPA (dissolved in 2 mL 200 proof ethanol) and 200 μL of β-mercaptoethanol (BME) in 0.1 M sodium tetraborate with 0.5% (g/mL) SDS, to make up a volume of 100 mL. The OPA reagent was kept in a dark bottle and used within 2 hours of preparation. Lysine was used to construct a standard curve (Figure 26, Appendix B). The blank used was 1% (g/mL) SDS. Each sample, standard, and blank (50 μL/well) was added into a 96-well clear, flat bottom polystyrene plate specific for UV readings (Corning, Inc., Corning, NY, USA). Subsequently, the OPA reagent (200 μL/well) was added and the plate was immediately placed in the microplate reader (BioTek Synergy HT, BioTek Instruments, Winooski, VT, USA). The plate was briefly shaken and held for 2 minutes at room temperature prior to reading the absorbance at 340 nm. Lysine contents (mg/mL) were corrected to percent lysine after dividing by the amount of protein (mg/mL). The original protein sample (stored at -20°C) for each type was used to represent day 0. Percent remaining free amino groups present in each sample, at each time point, a_w, and temperature, was determined (**Equation 6**).

Equation 6:

$$\%\ \text{Remaining Free Amino Groups} = 100 - \frac{100 * (\%\ \text{Lysine}_0 - \%\ \text{Lysine}_{\text{stored}})}{\%\ \text{Lysine}_0}$$

2.3.14. Protein Solubility and Aggregation

2.3.14.1. Quantitative Determination of Water Solubility

Before storage at 45°C, 50 mg protein of each of the three sample types—SPI, SPH, and 50/50—were weighed into 2 mL glass HPLC vials and stored, without caps, in eight plastic desiccators (Table 2) at 45°C. Samples were removed at days 28, 56, and 77 and stored at -40°C until they were solubilized and analyzed. Before solubilizing in 2 mL of DDW to make a 2.5% protein solution, the samples in the vials were weighed to assess

their gain in mass from day 0 of storage. This allowed for an approximation of increase or decrease in moisture content. The solubilized samples were vortexed and then shaken for one hour. After thorough mixing, 1 mL of each sample was removed to determine protein content by Dumas using the LECO® Nitrogen Analyzer (Section 2.3.5.). The remaining dispersions were then centrifuged at 15,682 x g for 10 minutes and the supernatants were removed for protein content analysis. To determine the protein solubility at each time point for each sample type, **Equation 7** was used.

Equation 7

$$\%\ \text{protein solubility} = \frac{(\text{original protein } \% - \text{supernatant protein } \%)}{\text{original protein } \%}$$

2.3.14.2. Qualitative Determination of Aggregation and Solubility

Samples prepared for percent solubility determination were also used for the qualitative determination of aggregation and solubility in various buffers. Aliquots (100 μL) of the sample dispersion and supernatant were retained for analyzing via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The remaining precipitate, obtained after dissolving in water and centrifuging, was dissolved in 1% SDS, thoroughly mixed, and centrifuged at 15,682 x g for 10 minutes. The supernatant was removed and the remaining precipitate was dissolved in 1% SDS (after which, a sample was taken for SDS-PAGE analysis) and 5% β-mercaptoethanol (BME); the solution was then centrifuged at 15,682 x g for 10 minutes and the supernatant was removed. Lastly, any remaining precipitate was again dissolved in 1% SDS and 5% BME, centrifuged, and the supernatant was removed. The procedure for solubilizing in various buffers and the codes of the resulting samples is outlined in Figure 4. The resulting eight samples, along with the original non-stored samples, were visualized using SDS-PAGE (section 2.3.14.3.). This was completed only for the 45°C samples stored at 45°C and at 0.59 and 0.79 a_w.

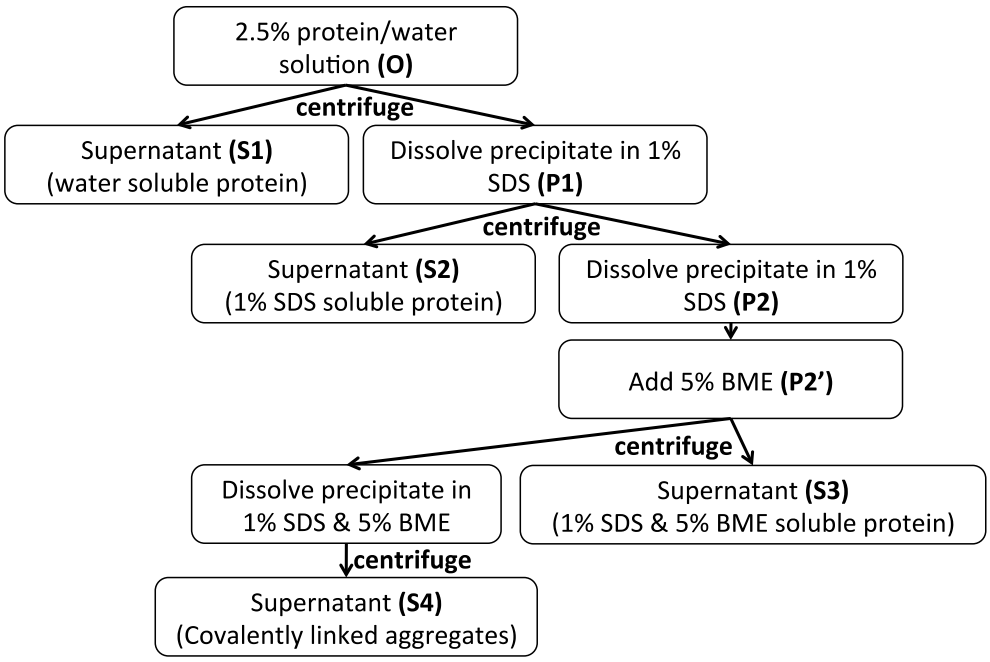

Figure 4. Solubilizing procedure to determine solubility and insoluble aggregates in various buffers after SDS-PAGE analysis.

2.3.14.3. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed following the method outlined by Laemmli (1970), with modifications. An aliquot (100 μL) of the each of the samples (Figure 4) was mixed 1:1 with Laemmli buffer and boiled for 5 minutes. SDS-PAGE was performed using Tris-Glycine TGX 4-20% pre-poured gradient gels (Bio-Rad Laboratories, Inc., Hercules, CA, USA). For each well, 7.5 μL of sample and 5 μL of a broad-range molecular weight marker (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was loaded. Gels were run using Tris-Glycine running buffer at ambient temperature first at 65 V for 25 minutes to pass through the separating phase and then run at 125 V for approximately 75 minutes or until completion. Gels were then stained with Coomassie Blue (45% methanol, 10% glacial acetic acid, 45% DDW, 3 g/L Coomassie Brilliant Blue R250) for 1 hour while shaking; then they were destained for another hour in 5% methanol, 5% acetic acid. Destaining solution was changed sever al times until the background was clear. Visualization of the gels was completed using a Nikon DX camera with the gel positioned over fluorescent lights for optimal resolution. Digital photographs were cropped and subsequently analyzed using Quantity One 1-D Analysis Software (Bio-Rad Laboratories, Inc.).

2.3.15. Determination of Fluorescent Compounds

The increase in fluorescence over time was analyzed to determine the effect of a_w and temperature on the rate of fluorescent Maillard compound formation. Fluorescence was determined at multiple time points in samples stored at 0.71-0.74 a_w (NaCl salt slurry) at all temperatures (25, 35, and 45°C); samples stored at 45°C and 0.05, 0.59, and 0.79 a_w were also analyzed. Fluorescent compounds formation was determined as outlined Rao *et al.* (2012b), with modifications. In triplicate, 10 mg of each sample was dissolved in 1 mL of DDW and vortexed. Pronase solution (0.178 mL of 20 U/mL in 50 mM Tris-HCl, pH 7.2), made fresh daily, was added to each sample, vortexed, and then incubated on a rotating mixer for 50 minutes at room temperature. The samples were then centrifuged at 15,682 x g for 15 minutes and the supernatant was removed. The supernatant was diluted 2x using phosphate buffered saline (PBS) solution (20 mM phosphate buffer with 15 mM NaCl, pH 7.0). An aliquot (200 µL) of each sample was added to a well of a black, opaque 96-well microplate (Corning, Inc., Corning, NY, USA) and the fluorescence intensity (FI) was measured at an excitation of 360 nm (bandwidth 40 nm) and emission of 460 nm (bandwidth 30 nm). For each sample type and a_w, the blank used was the corresponding sample type of the non-incubated protein batch kept at -20°C. All data were corrected to the FI of 5 µg/mL quinine sulfate solution (100 µg/mL quinine sulfate in 50 mM sulfuric acid), which was prepared fresh daily and diluted in PBS. To obtain the % FI/g protein, **Equation 8** was used.

Equation 8

$$\frac{\%\ \mathrm{FI}}{\mathrm{g\ protein}} = \frac{(\mathrm{Emi}_{stored} - \mathrm{Emi}_0)}{\mathrm{Emi}_{5\ ppm\ quinine\ sulfate}} \Big/ \mathrm{g\ protein}$$

Where:

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

2.3.16. Differential Scanning Calorimetry

The denaturation temperature (T_d) and change in enthalpy (ΔH) of soy protein isolate (SPI) stored at various a_w and 45°C over time were assessed using the differential scanning calorimeter (DSC) 1 STARe System (Mettler-Toledo International, Inc., Columbus, OH, USA). Protein dispersions were prepared by dissolving the stored powders in DDW to make a 20% protein solution (w/v). Samples were vortexed and shaken for 2 hours at room temperature prior to analysis. Using a positive displacement pipet, approximately 20 mg of the dispersion was weighed into an aluminum DSC pan, which was subsequently sealed and then held at 40°C for 10 min in the DSC. The run began at 40°C and ended at 110°C at a rate of 10°C/minute, and was followed by a second run to ensure complete denaturation. The sealed aluminum pans were weighed before and after to determine if there was any leakage; if there was, the thermogram was diregarded.

The T_d was determined by taking the first derivative of the DSC scan and correlating the point at which the slope was equal to zero (the lowest point of the endothermic peak) to the temperature. This was done for both the β-conglycinin peak and the glycinin peak. The ΔH was determined by integrating the peaks for β-conglycinin and glycinin separately and keeping the peak onset and endset consistent between runs and samples. The ΔH was corrected for the amount of protein present to aid in comparison between different moisture contents.

2.3.17. 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. Regression analysis was completed using Microsoft Excel to create 95% confidence intervals to determine significant differences between reaction rate constants. ANOVA tables for Chapter 2 can be found in Appendix L (Tables 11-19).

2.4. Results and Discussion

2.4.1. Intrinsic Properties and Moisture Sorption Isotherms of Soy Protein Powders

2.4.1.1. Intrinsic Properties of Soy Protein Powders Prior to Storage

Differences in the intrinsic properties (a_w, DH, and chemical composition) of SPI, SPH, and 50/50 were established prior to storage (Table 4). The moisture contents and a_w of SPI, SPH, and 50/50 were similar, as was expected, through limiting exposure to humid air prior to storage. To limit the development of bitter peptides and the decrease in functionality, the DH was kept below 8% (Matoba & Hata, 1972). This goal was achieved, as the SPH had an average DH of 6.07 ± 0.43%. The protein contents of all sample types were similar, ranging between 91.48 to 93.43%.

Table 4. Characteristics of soy protein powders utilized.

Samples	Protein (%)	Degree of hydrolysis (%)	Moisture content (%)	a_w	Total carbohydrate (%)	Glucose (μg/g)
SPI	93.43 ± 1.04	-	2.23 ± 0.65	0.08	2.95 ± 0.02	12.98 ± 1.30
SPH	91.48 ± 1.74	6.07 ± 0.43	2.40 ± 0.96	0.06	2.82 ± 0.08	15.48 ± 2.55
50/50	93.01 ± 0.72	2.04 ± 0.88	2.71 ± 0.84	0.09	2.91 ± 0.01	N/A

*Plus or minus standard error (n=3).

The total carbohydrate of each of the protein powders was less than 3%, and consisted mainly of stachyose, raffinose, sucrose, and glucose as determined using LC/MS (Figure 5, A & B). Note that the retention times were shown for stachyose, raffinose, and sucrose, while glucose was detected more precisely through zooming; glucose's retention time was approximately 34 minutes (Figure 5, C). The ESI-MS analysis was used to detect the sodium adduct forms of the sugars and define their type (Figure 26, Appendix F). Stachyose, raffinose, and sucrose are not reducing sugars because their anomeric carbons are part of glycosidic linkages, leaving no available hemiacetals or hemiketals to open and expose a reducing group.

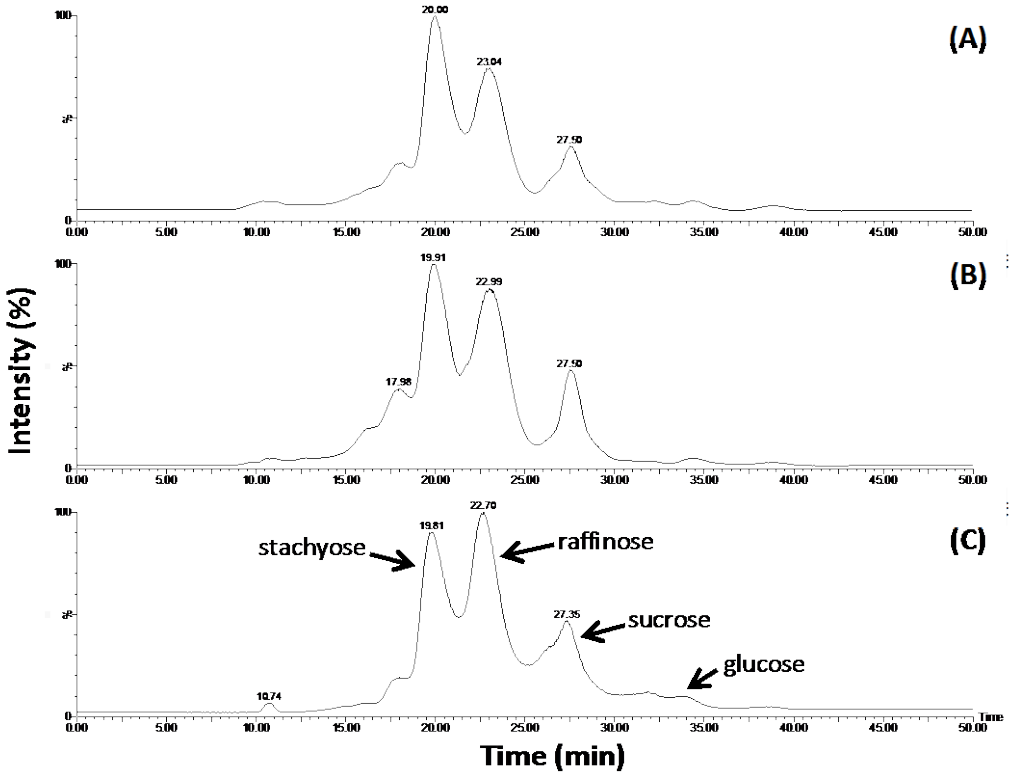

Figure 5. Chromatograms showing separation of extracted sugars in SPH (A), SPI (B), and the standard solution of stachyose, raffinose, sucrose, and glucose (C).

Because it was important to determine the content of reducing sugars, which could initiate the Maillard reaction, two methods were used to further assess the carbohydrate profiles of SPI and SPH. Using the DNS assay, percent reducing sugars of SPI and SPH was 0.47 ± 0.006% and 0.40 ± 0.007%, respectively. However, a downfall of this method is the lack of specificity for a particular monosaccharide and so the results only indicated the presence of reducing compounds without specifying reducing power. Reducing sugars vary in their reducing power and thus have different effects on the rate of the Maillard reaction (Laroque et al., 2008). Using an enzymatic assay, the glucose content of SPI and SPH was 12.98 ± 1.30 and 15.48 ± 2.55 ug/g, respectively. Because this amount was much smaller than the percentage found for overall reducing compounds in the DNS assay, it was hypothesized that other reducing sugars—besides glucose—were also present in SPI and SPH.

There is a general lack of information on the presence of monosaccharides in soy protein isolates in literature. Most carbohydrate analyses involved the use of acid hydrolysis to determine the types of monosaccharides present and/or percent total carbohydrate. Acid hydrolysis protocols would not allow for the determination of free monosaccharides originally present in the sample. Eldridge *et al.* (1979) determined that the main monosaccharides in SPI were ribose (0.19%), glucose (0.50%), galactose (0.51%), and mannose (0.75%). However, this was through the use of acid hydrolysis and so these sugars were not necessarily present as free monosaccharides, and thus may not have been available for chemical reactions. Also, the detected mannose was most likely derived from β-conglycinin, which is a glycoprotein containing mannose units. Although the carbohydrate analyses conducted in this work did not specify all the monosaccharides able to participate in the Maillard reaction in SPI and SPH, several conclusions can be drawn from the three methods employed. The LC/MS analysis indicated that the majority of sugars present in the SPI and SPH samples were sucrose, stachyose, and raffinose, which do not have reducing ends. The DNS assay results showed that there was a minimal amount of reducing sugars and/or compounds present at the beginning of storage. Finally, the enzymatic assay confirmed the presence of traces of glucose in the samples.

2.4.1.2. Protein/Peptide Profiles of SPI, SPH, and 50/50 Prior to Storage

The protein/peptide profiles were determined and examined for differences prior to the storage study (Figure 6). The greatest difference was between SPI and SPH due to the hydrolysis using Alcalase 2.4L. Alcalase is a serine alkaline protease produced by *Bacillus licheniformis* and contains subtilisin Carlsberg as its main enzyme component (Doucet et al., 2003). Alcalase has been shown to have greater specificity for β-conglycinin over glycinin (Hrckova et al., 2002; Lee et al., 1990; Zhao et al., 2013). This was substantiated through the observed reduction in the intensity of the protein bands corresponding to β-conglycinin subunits, while the protein bands corresponding to the glycinin subunits remained unchanged (Figure 6). There was also the appearance of more low molecular weight peptides and a general smearing in the SPH samples, which indicated the presence of new peptides of varying molecular weights. When visualizing the protein bands in lanes

corresponding to the O and S1 fractions of SPI, it was apparent that SPI was highly soluble in water as the protein bands in S1 had approximately the same intensity as those in O (Figure 6). Only faint bands corresponding to the β-conglycinin and glycinin subunits were seen in the P1 and S2 fractions of SPI (Figure 6), respectively. After adding BME, there were no bands observed in the P2' fraction of SPI (Figure 6). This proved that SPI was almost completely soluble in water (approximately 99% soluble, Figure 11, A) and was completely soluble in 1% SDS. Therefore, the SPI prepared had no aggregates or large polymers that could not be solubilized prior to storage.

Conversely, SPH had approximately 54% solubility in water (Figure 11, B), with many of the remaining proteins/peptides soluble in 1% SDS (P1 and S2 fractions of SPH, Figure 6). Sodium dodecyl sulfate disrupts non-covalent interactions between protein/peptide molecules. The greater solubility in SDS indicated that aggregate formation and resulting loss in water solubility was mostly attributed to hydrophobic interactions. Hydrolysis and thermal treatment, resulting in the unfolding of the protein, would cause exposure of hydrophobic residues that will take part in hydrophobic interactions and aggregate formation. The intensity of the band corresponding to the disulfide-linked acidic and basic subunits (AB-subunit) of glycinin was more intense in the P1 and S2 fractions than in the S1 fraction of SPH (Figure 6), indicating that the AB-subunits of glycinin were more soluble in 1% SDS than water. When the same fractions of SPI are compared (Figure 6), the AB-subunit is not even visible in the P1 and S2 fractions; this comparison between SPI and SPH indicated that during the production of SPH, the AB-subunits of glycinin were involved in hydrophobic interactions, most likely due to protein unfolding.

The 50/50 sample (approximately 71% soluble in water, Figure 11, C) reflected what was observed in the protein powders separately. More peptides and smearing were apparent in comparison to SPI, but less overall intensity was seen in the P1 and S2 fractions as compared to the same fractions of SPH. The 50/50 sample lanes also displayed more bands—mainly from the AB-subunits of glycinin (approximately 50-60 kDa)—present after dissolving the precipitate in 1% SDS as compared to SPI (P1 and S2 fractions of 50/50 compared to P1 and S2 fractions of SPI, Figure 6).

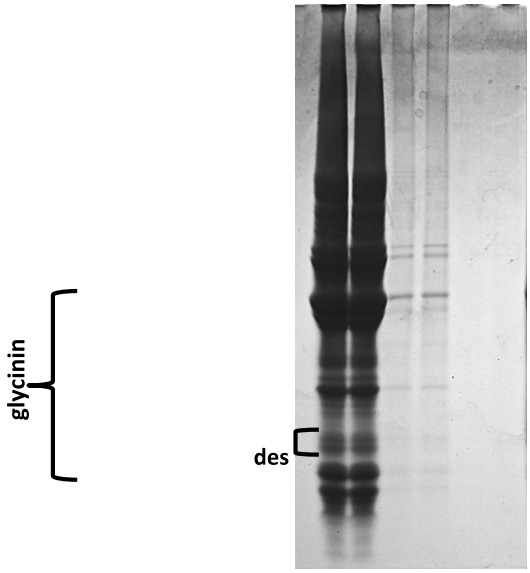

Figure 6. SDS-PAGE (under non-reducing conditions) visualization of the protein/peptide distribution in SPI, SPH, and 50/50 prior to storage. Molecular weights in kDa. Codes (O, S1, P1, S2, P2, and P2') are the same as those defined in Figure 4.

2.4.1.3. Moisture Sorption Isotherms of SPI, SPH, and 50/50

The determination of moisture sorption isotherms for each protein powder prior to storage aided in explaining how the isolate and hydrolysate differ in their absorption/adsorption of water at various RH. Using the DVS method on the AquaLab VSA, traditional static isotherms were created within 3 days rather than waiting approximately 2 months for a_w equilibration at room temperature. The points of equilibration with the RH and stabilization of the amount of weight gained were used to generate the GAB isotherms (Figure 7). The moisture sorption isotherms followed a Type II curve and had m_0 moisture values between 0.25 and 0.35 a_w. This means that chemical reactions needing water should not occur at or below this a_w or only occur at a very low rate.

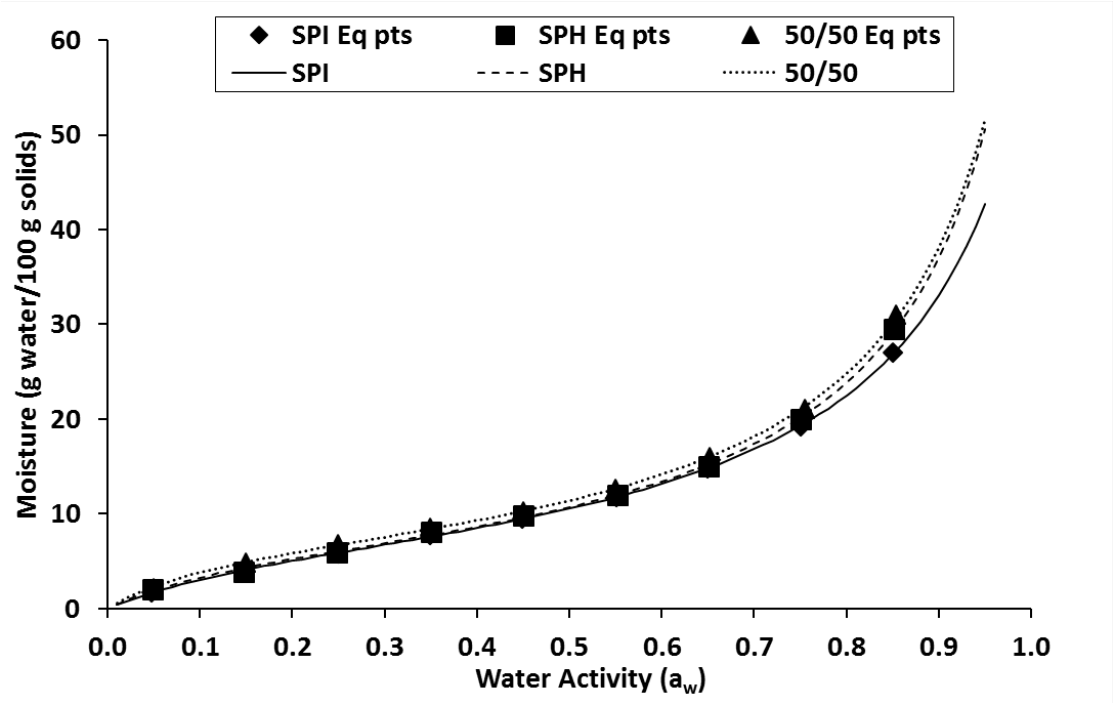

Figure 7. Moisture sorption isotherms of SPI, SPH, and 50/50 at 23°C. Eq pts are the points at which equilibrium to the RH were achieved.

The m_0 values for all three types of powders were similar, between 6.8 and 6.9 g H_2O/100 g solids (Table 5), which indicated that the hydrolysis did not expose new water adsorption sites of significance (Rao & Labuza, 2012). The m_0 moisture values were predicted to be at 0.32, 0.29, and 0.27 a_w for SPI, SPH, and 50/50, respectively. The *k* also was similar among all the isotherms, which indicated that the energy needed to adsorb water above the m_0 was similar for SPI, SPH, and 50/50. Lastly, the *C* was similar but slightly higher for SPH and 50/50 in comparison to SPI, which correlated to water bound more tightly in SPH and 50/50. However, the relative difference was very small in comparison to what has been found in other hydrolysates and their isolates, such as in dried egg white powders. Rao & Labuza (2012) observed a *C* of 32.5 and 11.9 for egg white isolate and hydrolyzed egg white, respectively. Based on our findings, it was expected that all of the soy protein powders investigated would adsorb water in a similar manner. Thus, physicochemical changes during storage would not be attributed to differences in water adsorption patterns, but rather to other inherent characteristics.

Table 5. Guggenheim-Anderson-de Boer (GAB) equation parameters of soy protein powders generated at 25°C.

Sample	Monolayer moisture value (m_0) (g H_2O/100 g solids)	Multilayer factor (k)	Surface heat constant (C)	MAPE[a]
SPI	6.9	0.89	6.85	1.34
SPH	6.8	0.92	8.00	3.15
50/50	6.9	0.91	9.99	1.82

[a] MAPE: mean absolute percentage error; MAPE values <5% indicate that GAB model had an excellent fit to the data (Labuza & Bell, 2000; Zhou & Labuza, 2007).

2.4.2. The Influence of Water Activity and Temperature on Color Change

2.4.2.1. Water Activity's Effect on Total Color Difference During Storage

Color change is one of several quality measures often used at manufacturing plants as a simple pass/fail criterion. However, color change can also indicate chemical changes such as those imparted by the Maillard reaction. Due to the trace amount of reducing sugars present in SPI and SPH, the extent of the Maillard reaction—if it were to occur—was expected to be low. When observing the total color difference (ΔE) rather than the individual color measurements, the change corresponding to a_w can be seen clearly. At 45°C, an increased a_w led to a significant ($P \leq 0.05$) increase in ΔE (Figure 8). A ΔE value of 1.0 is commonly considered the smallest color difference that can be detected by a trained specialist. However, when collecting samples, a ΔE value under 5.0 was difficult to visually discern in practice due to the variation of changes in all values: L^*, a^*, and b^*.

Soy protein isolate, SPH, and 50/50 stored at and above 0.33 a_w showed a significant ($P \leq 0.05$) increase in ΔE as compared to 0.05 a_w, which was the closest to the original a_w. It was expected that the color change would only begin to change significantly above 0.43 a_w, which was above the m_0 value. However, an increase in temperature can actually decrease the a_w of the m_0 (Labuza et al., 1985), which we had only determined for 25°C and not at 45°C. By the end of storage, there was not one sample type that consistently had a higher ΔE at every a_w level. This observation is attributed to the samples' similarities in intrinsic properties and isotherms prior to storage. However, at 0.59 and 0.71 a_w SPH

did have a significantly ($P \leq 0.05$) higher ΔE than 50/50 and SPI (Figure 8). Although the ΔE was statistically higher, it is important to note that the difference in the ΔE values for SPH with SPI and 50/50 were less than 5.0, meaning the increase was not highly impactful.

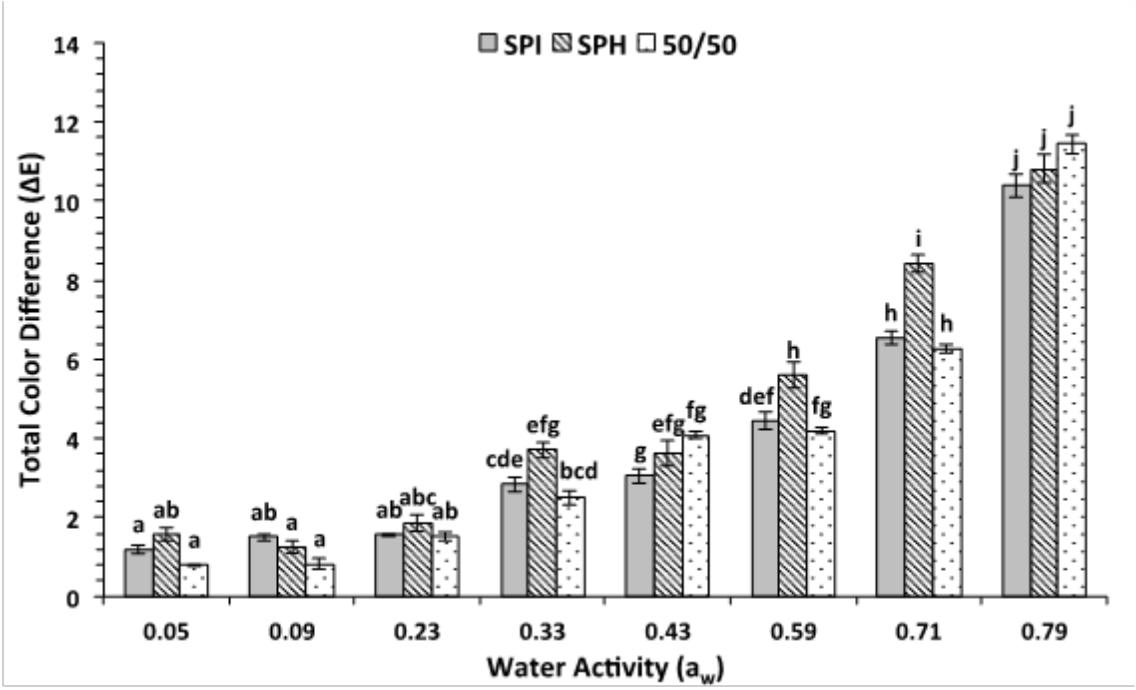

Figure 8. ΔE of SPI, SPH, and 50/50 after 84 days of 45°C storage at various a_w. Error bars represent standard error (n=3). Lowercase letters above the bars indicate significant difference between the means as determined following the Tukey-Kramer multiple means comparison test ($P \leq 0.05$).

The ΔE values do show a trend, but in comparison to other dry protein powder studies, the change is not as drastic. For example, storage of hydrolyzed egg white powder at 45°C and 0.79 a_w for 60 days experienced a ΔE over 30 (Rao et al., 2012b). The reason for soy protein's smaller ΔE in comparison to egg, for example, is most likely due to the smaller amount of reducing sugars—or sugars with less reducing power—present. Another reason could be related to the original color of the samples, which was yellow-tan rather than white, as is common for egg white and whey protein powders.

2.4.2.2. Changes in L^*, a^*, and b^* Values at 45°C Storage

During storage at all temperatures and a_w the change in L^*, a^*, and b^* values were measured to further track the differences in type of color change; however, only 0.79 a_w at

45°C had trend lines with a high goodness of fit ($R^2 > 0.85$). Storage at 25 and 35°C showed much smaller increments of change as compared to 45°C, which was expected, with only 0.74 a_w showing a suggestive trend of color change for L^* values (Figure 27, Appendix G). The reaction rates of the different values varied with their type: L^* values followed a pseudo-zero-order rate, a^* values followed a pseudo-first-order rate, and b^* values followed a pseudo-zero-order rate (Figure 9). However, only the L^* and b^* values had high goodness of fits ($R^2 > 0.75$) across all sample types. Although all sample types had similar ΔE values by the end of storage at 0.79 a_w, as previously discussed, the visual color and rate of change varied between sample types. Soy protein hydrolysate became darker and yellower throughout storage (lower L^* and higher b^*), while SPI became darker and redder (lower L^* and higher a^* value) by the end of storage. As expected, 50/50 performed in between SPI and SPH for all measured color values.

In color analysis, a decrease in L^* value (i.e. a decrease in lightness) can be used as an indicator of the Maillard reaction (Rao et al., 2012a; Rao et al., 2013c). However, this mainly functions as an indicator of the Maillard reaction's advanced glycation end products (AGEs), such as melanoidins, which display a brown color. The decrease in L^* value was not very high (Table 6), but there were some differences between sample types. Soy protein isolate and 50/50 had higher starting L^* values than SPH; but due to 50/50's higher reaction rate constant (k), 50/50 ended storage at a significantly ($P \leq 0.05$) lower L^* value than SPI. Soy protein hydrolysate had a significantly ($P \leq 0.05$) smaller k than SPI and 50/50. The differences in reaction rate constants and ending L^* values may have been largely due to their different starting L^* values. The L^* min for SPI was near SPH's L^* max, while 50/50's L^* min was near SPH's L^* min (Table 6).

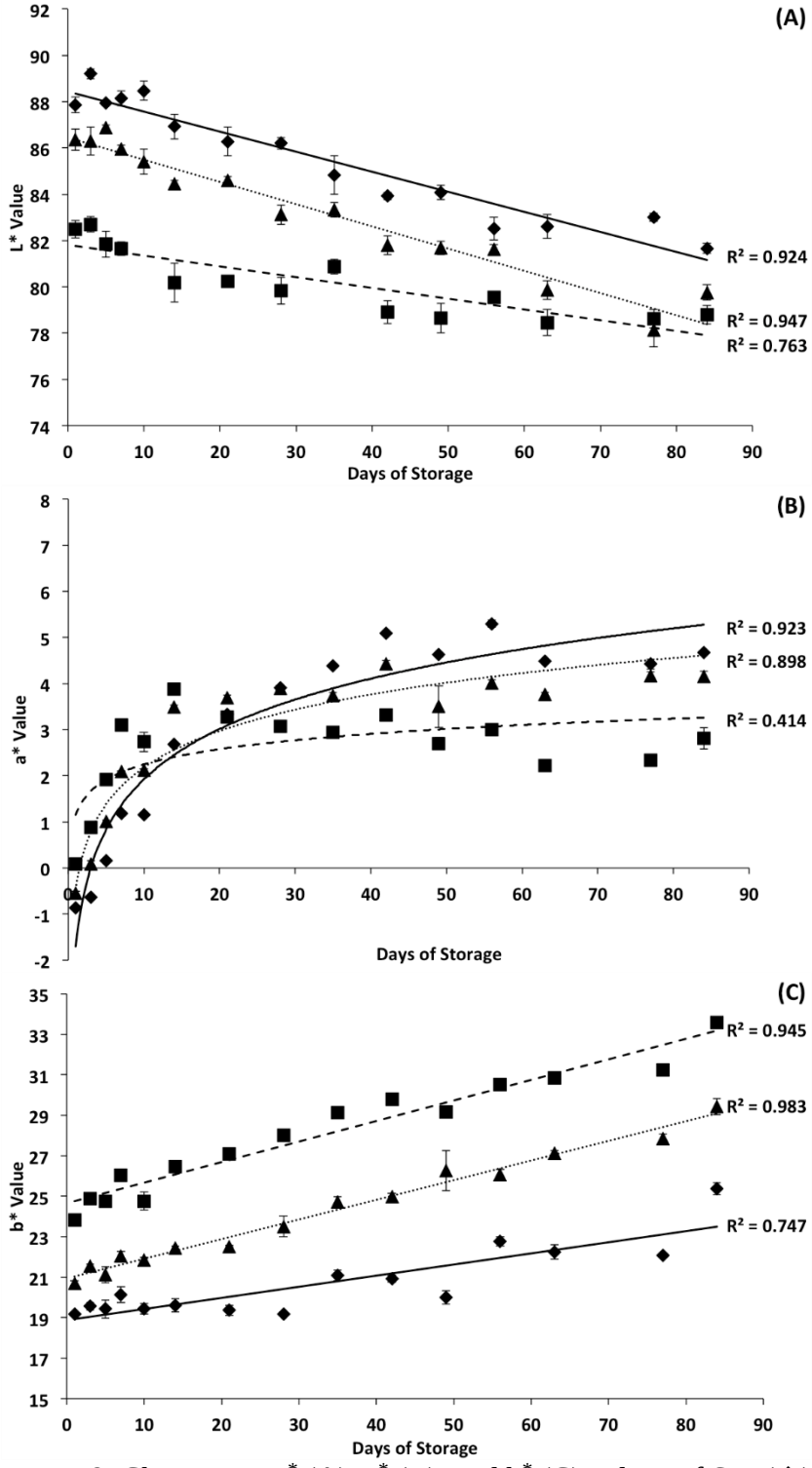

Figure 9. Changes in L^* (A), a^* (B), and b^* (C) values of SPI (◆), SPH (■), and 50/50 (▲) during storage at 0.79 a_w and 45°C. Error bars represent standard error (n=3). R^2 indicates goodness of fit for each trend line.

The L^* value decrease observed in all samples may have been due to a change in light reflection and therefore a physical reaction of water uptake—not a chemical reaction (Rao et al., 2012b). However, because the moisture sorption isotherms predicted similar water uptake (Table 6), the difference in L^* value *k* among samples types was probably not due to differences in amount of water uptake. Amounts of water uptake were verified to be similar through weighing of samples at the end of storage to approximate moisture content. The different *k* values may have been due to a combination of the Maillard reaction and physical water uptake. At higher a_w, the Maillard reaction will occur due to the greater amount and increased mobility of dissolved reactants (Rao & Labuza, 2012). The reflection of light for L^* values in SPI and 50/50 may have been more apparent because they started at a higher L^* value than SPH. Looking solely at the L^* values, this would indicate that 50/50 and SPI experienced a larger extent of the Maillard reaction. However, the changes in b^* values indicated that SPH may have experienced a larger extent of the Maillard reaction.

Table 6. Kinetic analysis of zero-order model for the change in L^* value as a function of sample type stored at 0.79 a_w and 45°C.

Apparent Linear Model	Sample Type		
	SPI	SPH	50/50
Best fit values			
L^* max	88.4[a]	81.8[b]	86.4[c]
L^* min	81.2[a]	77.9[b]	78.4[b]
k x 10^2 (day^{-1})	-8.7[a]	-4.7[b]	-9.6[a]
95% CI			
L^* max	87.8 – 89.1	81.1 – 82.5	85.9 – 87.0
L^* min	80.3 – 82.0	77.0 – 78.8	77.6 – 79.2
k x 10^2 (day^{-1})	-10.2 – -7.2	-3.0 – -6.3	-11.0 – -8.2
Goodness of fit			
R^2	0.924	0.763	0.947

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

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

Similarly to the L^* values, the increase in b^* value (increase in yellowness) also displayed a pseudo-zero-order model (Figure 9, C). The *k* values of SPH and 50/50 were

significantly ($P \leq 0.05$) higher than that of SPI (Table 7). Soy protein hydrolysate and 50/50 had a better goodness of fit (R^2) than SPI due to more consistent changes. Similar to the L^* value, the increase in b^* value could also have been due to the change in the reflection of light and not necessarily due to the Maillard reaction. Tracking b^* values is not typically used to indicate the Maillard reaction, but in these samples it seemed to trend well due to the yellow/tan color of soy protein rather than pure white, as is typically seen for milk and egg white protein powders. Combining the observed increase of b^* values to the findings from the other Maillard reaction indicators (increase in fluorescence and loss of free amino groups), suggested that SPH formed more Maillard reaction products than SPI and 50/50, as will be discussed further in Section 2.4.4.

Table 7. Kinetic analysis of zero-order model for the change in b^* value as a function of sample type for 0.79 a_w stored at 45°C.

Apparent Linear Model	Sample Type		
	SPI	SPH	50/50
Best fit values			
b^* max	23.5[a]	33.2[b]	29.1[c]
b^* min	18.9[a]	24.7[b]	20.9[c]
k x 10^2 (day^{-1})	5.5[a]	10.2[b]	9.7[b]
95% CI			
b^* max	22.4 – 24.6	32.3 – 34.0	28.7 – 29.5
b^* min	18.1 – 19.7	24.0 – 25.3	20.6 – 21.3
k x 10^2 (day^{-1})	3.6 – 7.4	8.7 – 11.6	8.9 – 10.5
Goodness of fit			
R^2	0.747	0.945	0.983

k x 10^2 (day^{-1}): reaction rate constant
[a,b,c] Values in the same row with different letters are significantly different ($P \leq 0.05$) by the use of a 95% confidence interval (CI).

2.4.3. Effect of Water Activity on Solubility and Aggregation During 45°C Storage

2.4.3.1. Changes in Water Solubility During Storage

The solubility of the various protein powders was examined solely at 45°C because the majority of visual changes (color change and stickiness/caking) occurred at this temperature in comparison to 25 and 35°C. Storage at 45°C is indicative of accelerated storage and provides information on what may happen during extended storage if time is a

constraining variant. As hypothesized, storage at higher a_w led to the formation of insoluble aggregates more so than at lower a_w (Figure 10). However, the effects were different for SPI, SPH, and 50/50.

Soy protein isolate had the highest significant ($P \leq 0.05$) change in % solubility among the three protein powders. After 77 days of storage, there was over a 40% drop in solubility at 0.33 a_w, which is at the m_0 (Figure 10, A). However, because higher temperatures have been shown to shift the m_0 to a lower value (Labuza et al., 1985), the drop in solubility was most likely above the actual m_0 at 45°C. A significant ($P \leq 0.05$) drop in % solubility at 0.33 a_w was also observed in 50/50 (Figure 10, C), however, it was less severe with a decrease of 24.5%. For SPH, % solubility at 0.33 a_w remained similar to that of the original (Figure 10, B). Among the three powders, SPI had the largest decreases in % solubility, with 21.1%, 13.5%, and 17.5% solubility observed after 77 days of storage at 0.59, 0.71, and 0.79 a_w, respectively. Soy protein hydrolysate had the smallest losses in % solubility, with 41.7%, 31.1%, and 33.7% solubility observed after 77 days of storage at 0.59, 0.71, and 0.79 a_w, respectively. The trend of change in % solubility of 50/50 fell in between that of SPI and SPH, but was closer to that of SPI, with 39.9%, 18.5%, and 25.1% solubility observed after 77 days of storage at 0.59, 0.71, and 0.79 a_w, respectively.

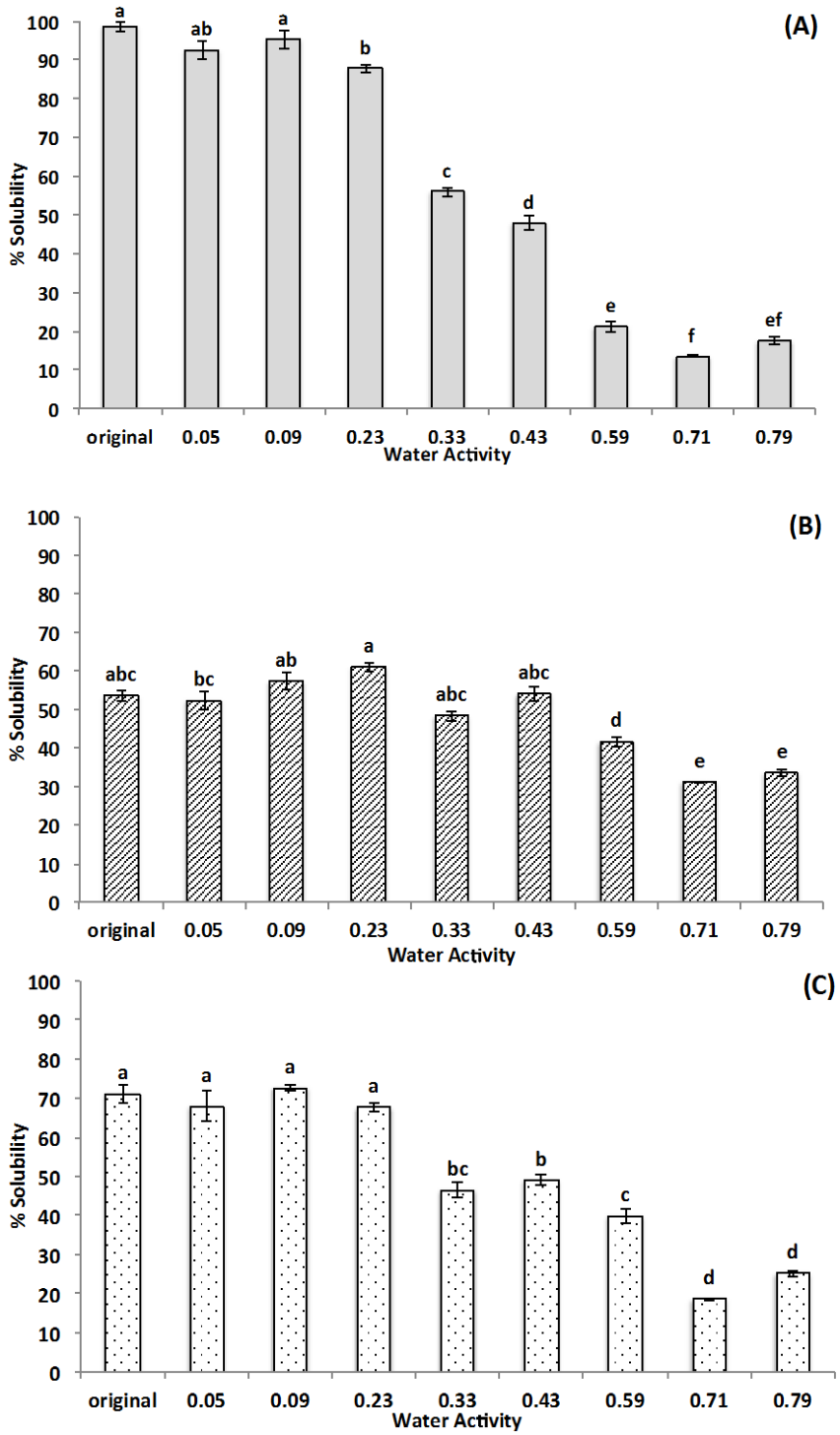

Figure 10. % Solubility of SPI (A), SPH (B), and 50/50 (C) at 45°C after 77 days of storage at various water activities as compared to the solubility prior to storage (original). Error bars represent standard error (n=3). Lowercase letters above the bars indicate significant difference between the means as determined following the Tukey-Kramer multiple means comparison test ($P \leq 0.05$).

The most drastic losses in % solubility were observed after 77 days, but for some a_w there was a steep decrease after only 28 days of storage. For SPI, 28 days of storage at 0.59 a_w led to a solubility of 58.2%, and then by 56 days had a significantly ($P \leq 0.05$) lower solubility of 22.9%, which did not change for the duration of the storage study (Figure 11, A). The same trend was observed for the SPI samples stored at 0.71 and 0.79 a_w, although the decrease in % solubility was only significant ($P \leq 0.05$) at 0.79 a_w. Interestingly, neither SPH or 50/50 had a significant ($P \leq 0.05$) change in solubility after 28 days of storage at 0.59, 0.71, and 0.79 a_w (Figure 11, B, C). For all sample types, the original solubility was maintained at 0.05 a_w, indicating that the presence of moisture was the driving force of aggregation during storage.

The decreased protein solubility at higher a_w was most likely due to the increased mobility of protein molecules, leading to the formation of intermolecular covalent and/or noncovalent bonds (Zhou & Labuza, 2007). Studies done on aggregation due to thermal treatment and pH change have shown that β-conglycinin and β-conglycinin-derived peptides may inhibit glycinin aggregation (Kuipers et al., 2006; Guo et al., 2012). In our study, the use of Alcalase formed β-conglycinin-derived peptides. Therefore, SPH's retention of % solubility during storage, as compared to SPI's decrease in % solubility, may have been due to the presence of β-conglycinin-derived peptides that inhibited aggregation during storage. The thermal treatment applied during production of SPH, however, most likely contributed to the lower solubility compared to SPI prior to storage. Further research is needed to gain a better understanding of the aggregation kinetics of SPI versus SPH.

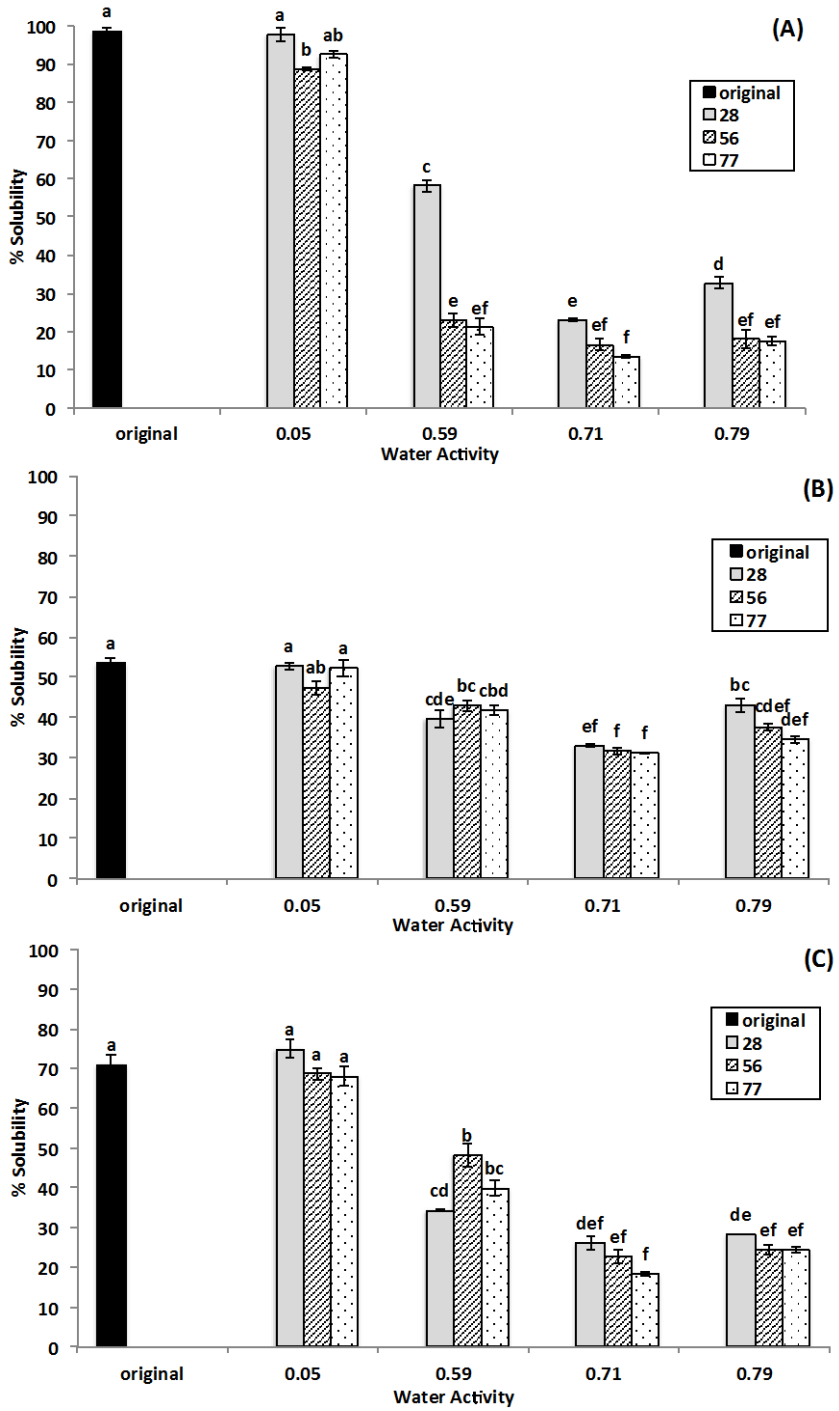

Figure 11. % Solubility at 28, 56, and 77 days at selected water activities for SPI (A), SPH (B), and 50/50 (C) at 45°C as compared to % solubility prior to storage (original). Error bars represent standard error (n=3). Lowercase letters above the bars indicate significant difference between the means as determined following the Tukey-Kramer multiple means comparison test ($P \leq 0.05$).

2.4.3.2. Effect of Water Activity on Aggregation Patterns During Storage

To further elucidate the types of interactions causing the formation of insoluble aggregates upon storage at high a_w, SDS-PAGE was performed to examine the protein/peptide molecular weight profile. The changes in protein/peptide profile were compared among samples stored for 0 (original samples kept at -20°C for the duration of the storage), 28, and 77 days at 0.59 and 0.79 a_w. Samples stored at 0.59 and 0.79 a_w were chosen because of the large losses in percent solubility observed. Those stored at 0.71 a_w were not included because their percent solubility was not statistically different from that of the samples stored at 0.79 a_w. Note that day 56 was used in place of day 77 for storage of 50/50 at 0.59 a_w because the day 77 sample was destroyed. Days 56 and 77 were not statistically ($P \leq 0.05$) different in terms of solubility (Figure 11, C).

The original SPI sample was almost completely soluble in water, with only very faint bands in the supernatant (S2, original) observed after SDS was added to the precipitate (Figure 12, A). After 28 and 77 days of storage at 0.59 a_w, the bands in the S2 fraction had a greater intensity than those present in the S2 fraction of the original (Figure 12, A). Adding 1% SDS disrupted the hydrophobic interactions that had formed to create insoluble aggregates. For the S2 fraction, the most prominent changes in band intensity between the original samples and those stored at 28 days correlated to α' (72 kDa), α (70 kDa), and the AB-subunit (50-60 kDa) of glycinin (Figure 12, A). After the addition of BME, faint bands were observed in the P2' and S3 fractions of the SPI sample stored for 28 days, corresponding to the acidic (~40 kDa) and basic polypeptides (~20 kDa) of glycinin (Figure 12, A). The addition of BME broke disulfide (S-S) linkages, which is why the acidic and basic polypeptides in glycinin were visible as separate bands. After 77 days of storage, similar observations were noted, yet with fainter bands observed in the S1 fraction and darker bands observed in the P1 and S2 fractions (Figure 12, A). This observation correlated well with the higher reduction in solubility observed after 77 days as compared to 28 days of storage at 0.59 a_w (Figure 11, A).

The original SPH sample was approximately 50% soluble in water (S1), with the remaining precipitate (P1) almost completely soluble in 1% SDS (S2) (Figure 12, B, original). After the addition of BME to break the disulfide bonds, faint bands corresponding

to the acidic (~40 kDa) and basic polypeptides (~20 kDa) of glycinin were present in the P2' fraction of the original SPH sample (Figure 12, B). After 28 and 77 days of storage at 0.59 a_w, no noticeable difference was apparent between the stored samples and the original sample. Although there was a significant ($P \leq 0.05$) difference in % solubility of the original SPH and that stored for 28 and 77 days at 0.59 a_w (Figure 11,B) insoluble aggregates were not detected through SDS-PAGE visualization.

The pattern of aggregation for 50/50 was similar to that of SPI. After 28 and 56 days of storage at 0.59 a_w, the loss in % solubility was evident by the observed increase in band intensity in the O fraction versus the S1 fraction (Figure 11, C). Greater band intensities corresponding to the AB-subunit and β-conglycinin subunits were observed in the P1 and S2 fractions of the sample stored for 28 and 56 days as compared to the original (Figure 11, C). This observation indicated that the formation of insoluble aggregates was due mainly to hydrophobic interactions.

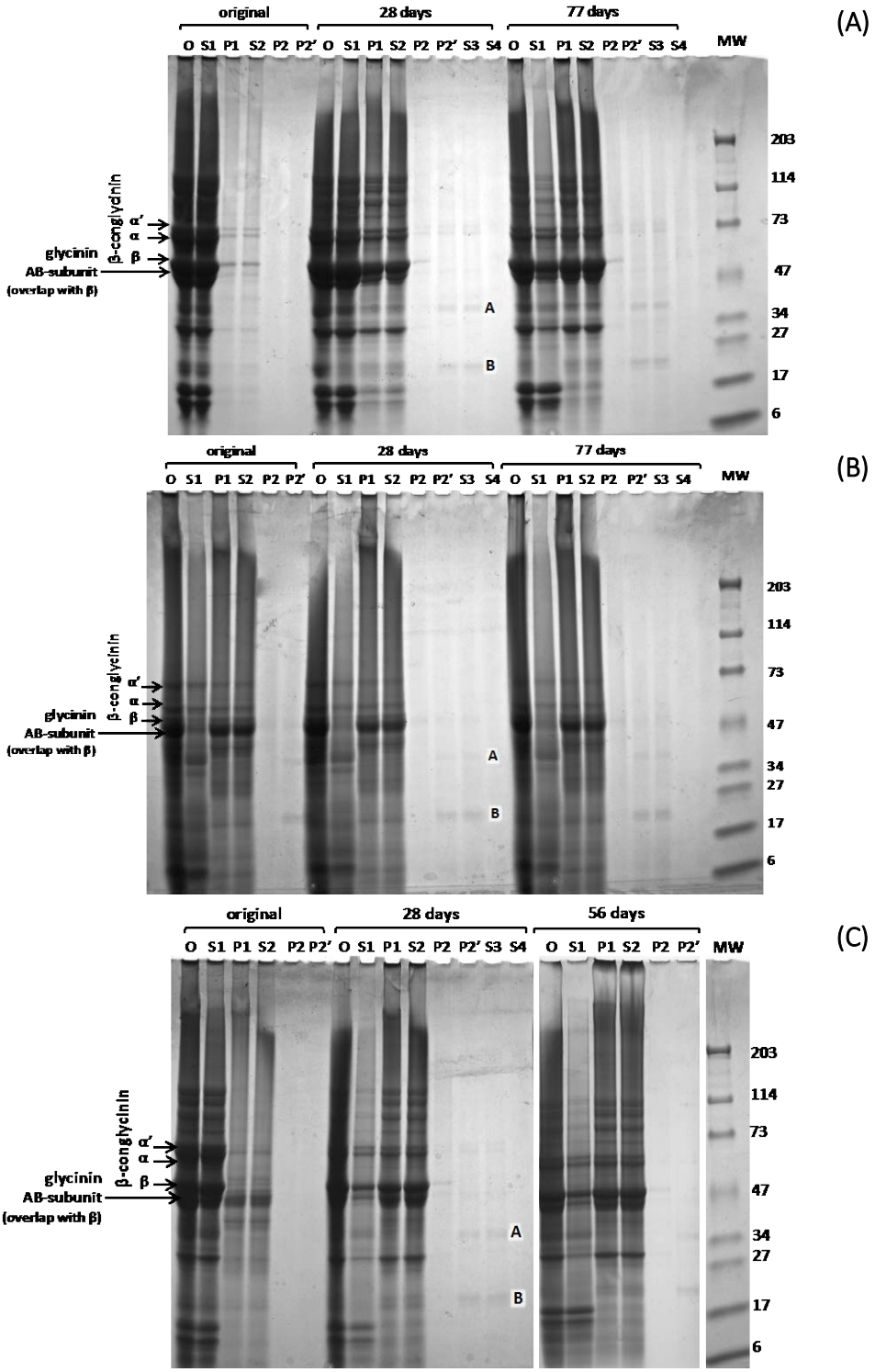

Figure 12. Visualization of change in protein/peptide distribution after storage at 0.59 a_w and 45°C after 28 & 77 days for SPI (A) and SPH (B), and after 28 & 56 days for 50/50 (C). Molecular weights in kDa. Glycinin polypeptide bands: acidic (**A**) and basic (**B**). Codes (O, S1, P1, S2, P2, P2', S3, and S4) are the same as defined in Figure 4.

The greatest changes in protein/peptide aggregation were observed in the samples stored at 0.79 a_w (Figure 13). The decrease in % solubility of SPI from day 0 to day 28 and 77 could be visualized by comparing the S1 fractions (Figure 13, A). The disappearance of the β-conglycinin subunits and the glycinin AB-subunit was observed when comparing the S1 fraction of the sample stored for 28 days to that of the sample stored for 77 days (Figure 13, A). Apparently, these subunits must have been involved in the formation of insoluble aggregates over time. The aggregates at 77 days were formed through hydrophobic interactions (S2), as was observed after disruption with 1% SDS; through disulfide linkages (S3), as was observed after reduction with BME; and through covalent linkages (S4), as was observed by the dark high molecular weight bands at the top of the gel, corresponding to the large polymers that were unable to enter the gel (Figure 13, A). It is hypothesized that the formation of the large polymers unable to enter the gel were induced by the Maillard reaction, which will be further discussed in Section 2.4.4. Others have also used this observation to confirm the presence of high molecular weight Maillard reaction products after dissolving stored egg protein samples in both SDS and a reducing agent (Rao et al., 2012a, 2013a).

Although SPH had smaller decreases in % solubility after 28 and 77 days of storage at 0.79 a_w as compared to SPI (Figure 11, A & B), overall band lightening in the S1 fraction was visualized (28 & 77 days, Figure 13, B). The same types of molecular interactions that lead to aggregation in SPI—hydrophobic interaction and disulfide linkages—were observed in SPH. High molecular weight polymers were not observed in the S4 fractions (Figure 13, B); however, it is possible that there was Maillard-induced aggregation but that the polymers formed were small enough to still enter the gel.

50/50 had a similar aggregation pattern to that of SPI and SPH. Although the difference in % solubility was not significant ($P \leq 0.05$) between the samples stored for 28 and 77 days at 0.79 a_w (Figure 11, C), bands larger than 17 kDa in the S1 fraction became fainter after 77 days compared to those in the S1 fraction stored for 28 days (Figure 13, C). Because the formation of aggregates was not greater than that observed for SPI, peptides formed during hydrolysis most likely did not act as promoters of aggregation. However,

without being able to specifically track the low molecular weight peptides, it is unknown whether they participated in the aggregation.

Soy protein isolate's loss of % solubility during storage at elevated a_w levels was in agreement with other studies. Martins & Netto (2006) observed a disappearance of the β subunits of β-conglycinin and acidic polypeptides of glycinin in SPI after 90 days of storage at 45°C and 0.74 a_w. By the end of storage, 120 days at 45°C, only the basic polypeptides of glycinin were apparent in SDS-PAGE gels. Significant losses in solubility were also observed by Hou & Chang (2004a, 2004b) for purified fractions of β-conglycinin and glycinin after storage at 84% RH and 30°C for 8 months. In our study, both β-conglycinin and glycinin subunits were involved in the formation of insoluble aggregates. Glycinin was mainly involved in disulfide linkages but also took part of hydrophobic interactions (Figure 13). The subunits of β-conglycinin seemed to be involved in the formation of thiol-disulfide interchange, as they remained partly insoluble until BME was added [as seen in fractions P2' and S3 (Figure 13, A)], even though the amount of free surface sulfhydryl groups is reportedly small for β-conglycinin. However, β-conglyinin seemed to be more involved in hydrophobic interactions across sample types as greater band intensities corresponding to β-conglycinin subunits were observed in the S2 fraction than the S3 fraction (Figure 13).

Because of the large extent of aggregation in SPI, it was at first assumed that the proteins had become denatured after storage at high a_w. However, DSC measurements of SPI stored at 0.79 a_w for 56 days had no significant ($P \leq 0.05$) change enthalpy (ΔH) as compared to an equilibrated (stored for 5 days at 0.79 a_w) sample (Figures 33 and 34, Appendix I). If the protein had become denatured, the ΔH would have been smaller than the equilibrated sample's ΔH. Overall, it is hypothesized that the hydrophobic interactions and disulfide bonds formed over time were the result of liquid bridges (Aguilera et al., 1995) and greater mobility of protein molecules to allow for noncovalent interactions and covalent bonds. Similar findings for moisture-induced aggregation in whey and egg white proteins by Zhou & Labuza (2007), Zhou *et al.* (2008b), and Rao & Labuza (2012) support this hypothesis.

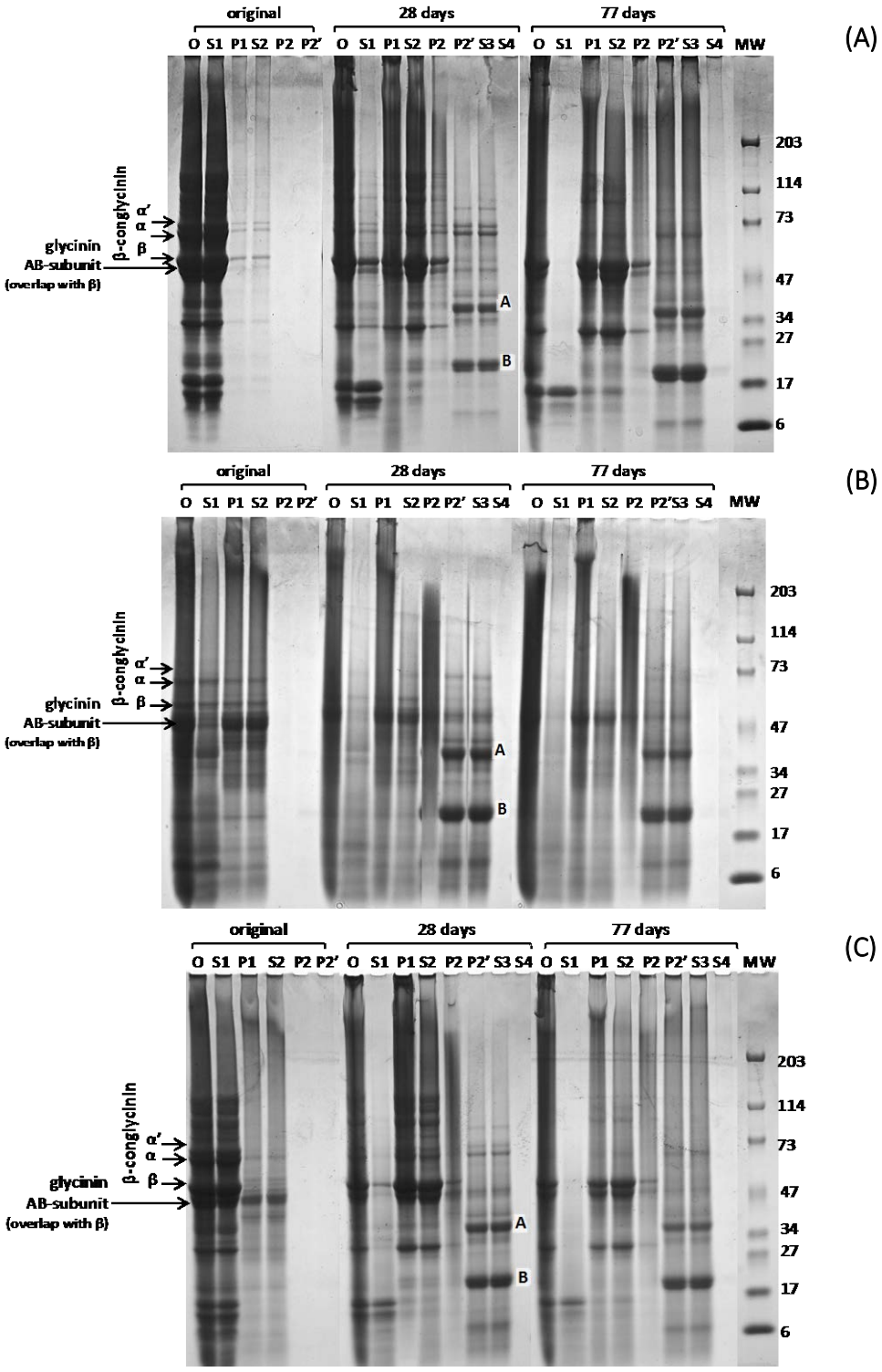

Figure 13. Visualization of change in protein/peptide distribution after 28 & 77 days of storage at 0.79 a_w and 45°C for SPI (A), SPH (B), and 50/50 (C). Molecular weights in kDa. Glycinin polypeptide bands: acidic (**A**) and basic (**B**). Codes (O, S1, P1, S2, P2, P2', S3, and S4) are the same as defined in Figure 4.

2.4.4. Effect of Temperature and Water Activity on Loss of Free Amino Groups and Formation of Fluorescent Maillard Reaction Products

2.4.4.1. Change in Free Amino Groups During Storage

Analyzing the loss of free amino groups provides a good indication of the Maillard reaction even before brown pigments are observed (Labuza & Massaro, 1990; Warren & Labuza, 1977). The primary amino group involved in the Maillard reaction is often the ε-amino group of the amino acid, lysine. The OPA method, however, was used to assess the overall loss of free amino groups rather than focusing on % loss in the ε-amino group of lysine alone. While the ε-amino group of lysine is highly reactive, other amino groups such as terminal amines and the guanidinium group of arginine and imidazole group of histidine can be involved in the Maillard reaction (Ajandouz & Puigserver, 1999).

No consistent zero- or first-order reaction was observed for the loss of free amino groups over time at any of the a_w levels and temperatures studied. However, the most consistent trends, with significant ($P \leq 0.05$) differences over the course of storage time, were seen at 0.79 a_w and 45°C for all samples stored (Figure 14). Both SPH and 50/50 had significant ($P \leq 0.05$) losses in % free amino groups within the first 2 weeks of storage, while SPI took approximately 4 weeks to show a significant loss. By 77 days of storage, all sample types had a significant loss of free amino groups with 74.3%, 82.3%, and 79.3% remaining free amino groups for SPI, SPH, and 50/50, respectively. After having a significant ($P \leq 0.05$) loss within the first month of storage, there were no significant changes for the remainder of the storage time. Findings were similar to Labuza & Massaro (1990), Wolf et al. (1977a), and Wolf et al., (1977b), where amino acid loss initially followed pseudo-first-order kinetics, and then had a no-loss or recovery period.

In contrast to the high a_w of 0.79, there was no significant ($P \leq 0.05$) change in percent remaining free amino groups at 0.05 a_w across all temperatures and sample types (Figure 31, Appendix H). Conversely, the majority of the studied a_w and temperatures produced more complicated trends (Figures 28-32, Appendix H). After 77 days of storage at 0.71 a_w and 45°C, there was no significant ($P \leq 0.05$) change in percent remaining free

amino groups for SPI, a significant drop to 78.6% remaining free amino groups for SPH, and a significant drop to 86.9% remaining free amino groups for 50/50 (Figure 29, Appendix H). At 0.59 a_w and 45°C, SPI experienced a significant ($P \leq 0.05$) drop in % remaining free amino groups within the first week and then increased significantly above the original amount of free amino groups by 42 days of storage (Figure 30, A, Appendix H). At the same parameters, SPH and 50/50 experienced losses, gains, and periods of stabilization of free amino groups throughout storage (Figure 30, B & C, Appendix H). The extent of change in % remaining free amino groups was lower in samples stored at 25°C and 35°C in comparison to those stored at 45°C. This observation is attributed to higher reaction rates at higher temperatures. Unexpectedly, the largest losses at 25°C were observed at 0.54 a_w instead of 0.74 a_w (Figure 32 & 33, Appendix H); while for the majority of a_w treatments at 35°C, the variances were too large to statistically compare losses over time (data not shown).

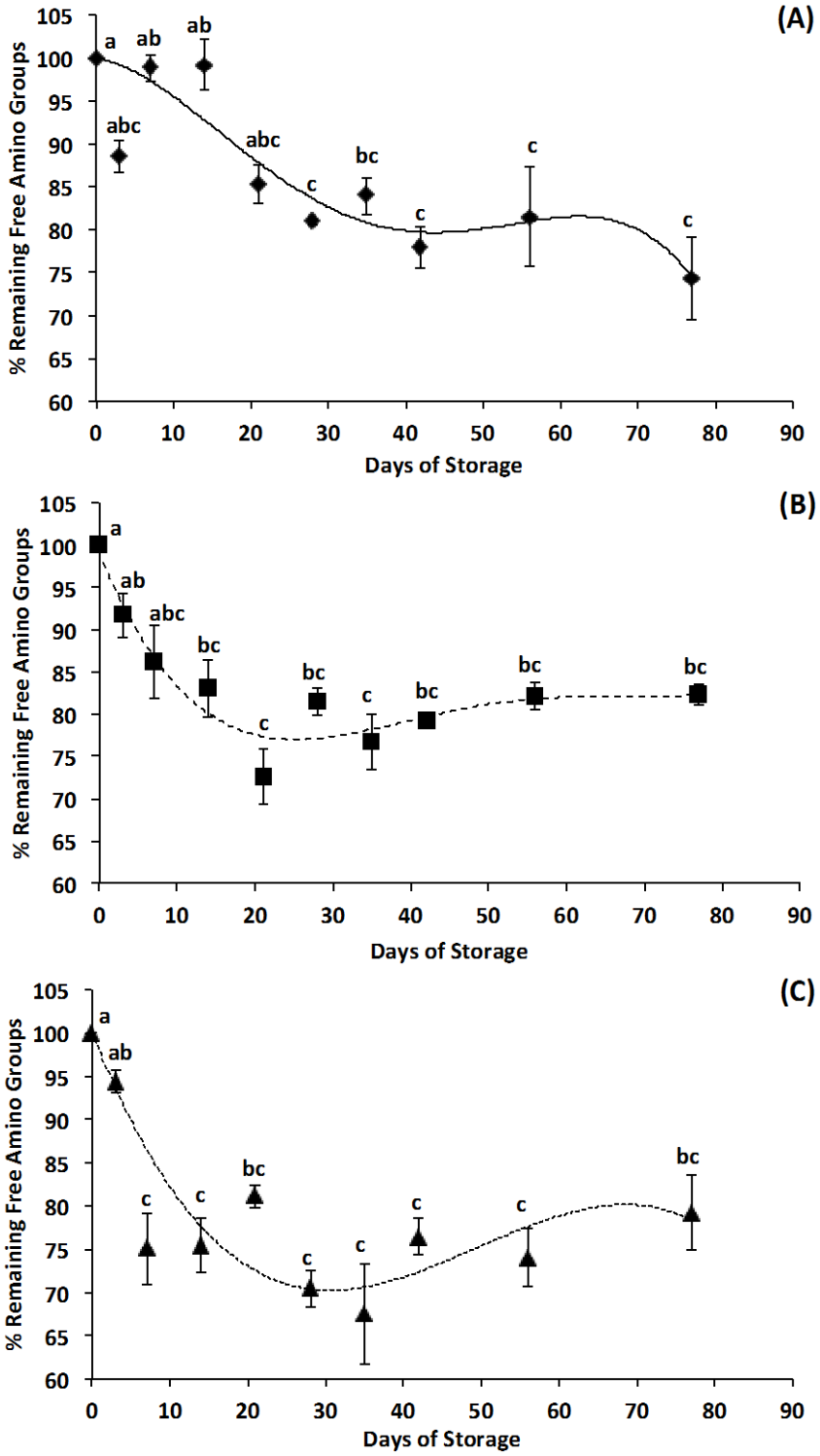

Figure 14. Change in % free amino groups in SPI (A), SPH (B), and 50/50 (C) during storage at 0.79 a_w and 45°C. Error bars represent standard error (n=3). Lowercase letters above each point indicate significant difference between the means as determine following the Tukey-Kramer multiple means comparison test ($P \leq 0.05$).

There are several explanations for the inconsistent changes in % remaining free amino groups. Firstly, the beginning stage of the Maillard reaction, when the Schiff base is formed, is reversible (Hodge, 1953). Therefore, reducing sugars may react with free amino groups and then disassociate. Additionally, Amadori rearrangement products during the intermediate stage of the Maillard reaction can regenerate free amino groups through fission, deamination, and/or dehydration reactions (Baisier & Labuza, 1992; Labuza & Massaro, 1990; Wolf et al., 1977). Therefore, it is quite common to see a decrease and then an increase in percent remaining free amino groups. Secondly, there may have been endogenous or microbial proteases present, which could cleave peptide bonds and thus release more free amino groups during storage. Baisier & Labuza (1992) showed that an increase in amino acid concentration (with a constant reducing sugar content) resulted in an increase in the rate constant of the loss of glycine, an amino acid. Therefore, the presence of microbial proteases could also explain the observed variation in rates of gains, losses, and stabilization of percent remaining free amino groups. Lastly, reaching equilibrium at a specific a_w in the desiccators took longer at temperatures below 45°C (especially when opening and closing desiccators often to remove samples), thus extending the time to have significant changes in percent remaining free amino groups and resulting in greater sample variation.

2.4.4.2. Increase in Fluorescence Over Time

It is widely accepted that the initial stage of the Maillard reaction does not result in fluorescence. However, Amadori rearrangement in the intermediate stage gives rise to colorless reductones and fluorescent compounds (Friedman & Kline, 1950; Hodge, 1953; Morales & Boekel, 1997). Studies have shown that fluorescent compounds may convert to AGEs and also that AGEs can be fluorescent (Tessier et al., 2002; Matiacevich et al., 2005). To monitor the extent of the Maillard reaction into the different stages (beginning, intermediate, or advanced), and the formation of related products, the amount of fluorescence intensity (%FI) over time was analyzed.

The formation of fluorescent compounds followed a pseudo-zero order reaction rate for all sample types (Figure 15; Figure 35, Appendix J). The %FI increased with time for

all samples stored at various a_w and temperatures, but had significantly different reaction rate constants (*k*). Although a pseudo-zero-order model fit the progression of %FI for all sample types ($R^2 \geq 0.949$) at $a_w \geq 0.59$, a decrease in the steepness of the slope over time for SPH was observed. Conversely, for SPI and 50/50 the %FI continued to increase throughout storage at 45°C (Figure 35, Appendix J). Soy protein hydrolysate's decrease in slope steepness could be because SPH began storage with more free amino groups that SPI and 50/50, and thus saturation of reducing compounds with free amino groups occurred more rapidly. The reduction in slope steepness also suggested that more of the Maillard reaction products were in the intermediate and advanced stages in SPH than in SPI and 50/50.

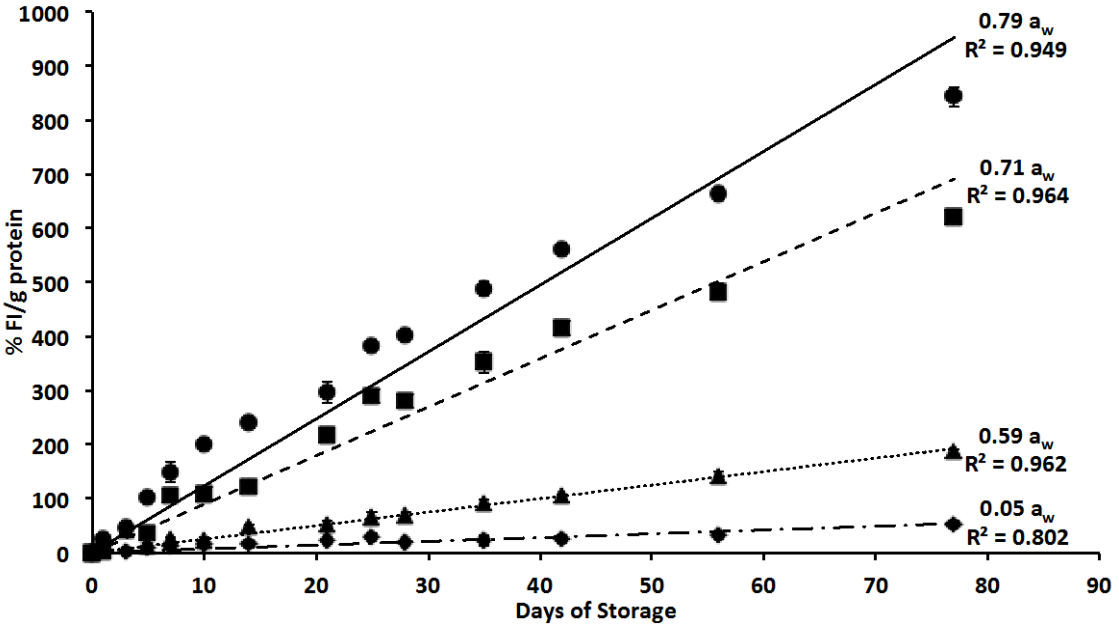

Figure 15. Change in fluorescence intensity (%FI) per gram protein over time at 45°C storage for SPH at 0.05, 0.59, 0.71, & 0.79 a_w. Error bars represent standard error (n=3). Goodness of fit (R^2) indicated for each regression.

When comparing the *k* values and %FI maxima for different sample types stored at 0.59, 0.71, and 0.79 a_w, a clear trend was evident (Table 8). There were significant differences ($P \leq 0.05$) in %FI maxima reached after 77 days of storage and *k* among samples stored at different a_w as modeled by zero-order reaction kinetics. However, when comparing *k* for sample types at the same a_w, *k* values were similar at 0.59 a_w, significantly

different ($P \leq 0.05$) at 0.71 a_w, and at 0.79 a_w were significantly lower in SPI than in SPH and 50/50. The %FI maxima were only significantly different at 0.71 a_w. Because reducing sugar levels were low to start with, it is possible that other reactants may have played a role in the progression of the Maillard reaction.

Table 8. Reaction rate constant (*k*) and percent fluorescence intensity maximum (%FI max) of samples stored at 45°C for 77 days.

Sample	*k* (day^{-1})			%FI max		
Type	0.59	0.71	0.79	0.59	0.71	0.79
SPI	1.45^{ax}	4.97^{bx}	10.49^{cx}	111.65^{ax}	382.69^{bx}	881.16^{cx}
SPH	2.50^{ax}	8.98^{by}	12.38^{cy}	192.50^{ax}	691.46^{by}	953.26^{cx}
50/50	1.68^{ax}	7.12^{bz}	11.65^{cy}	129.36^{ax}	548.24^{bz}	897.05^{cx}

[a,b,c] Values in the same row with different letters are significantly different ($P \leq 0.05$) as determined through using a 95% confidence interval.
[x,y,z] Values in the same column with different letters are significantly different ($P \leq 0.05$) as determined through using a 95% confidence interval.

To determine the effect of temperature on %FI, *k* values across different temperatures were compared at 0.71-0.74 a_w (NaCl saturated salt solution). Soy protein hydrolysate had a significantly higher ($P \leq 0.05$) *k* than SPI and 50/50 across all temperatures (Table 9). An Arrhenius plot using the different *k* values at 0.71-0.74 a_w was made (Figure 16) to determine the activation energy (E_A) of each sample type. SPI and 50/50 had similar E_A, while SPH had the lowest value. From the higher reaction rates and lower E_A we can conclude that SPH had a higher reactivity than SPI, which resulted in the formation of more fluorescent compounds. Also, all samples' E_A fell within the range for the Maillard reaction of 60-160 kJ/mole (van Boekel, 2001). The high E_A experienced suggests that the reaction is very sensitive to temperature changes (Bell, 2007).

Table 9. Reaction rate constant (k) and activation energy (E_A) of samples stored at 0.71-0.74 a_w (NaCl saturated salt solution).

Sample Type	k (day^{-1})			E_A (kJ/mole)
	25°C	35°C	45°C	
SPI	0.27^{ax}	4.97^{bx}	5.21^{bx}	118.06
SPH	0.69^{ay}	6.74^{by}	8.98^{by}	102.18
50/50	0.35^{ax}	5.91^{bz}	7.12^{cz}	119.72

[a,b,c] Values in the same row with different letters are significantly different ($P \leq 0.05$) as determined through using a 95% confidence interval.

[x,y,z] Values in the same column with different letters are significantly different ($P \leq 0.05$) as determined through using a 95% confidence interval.

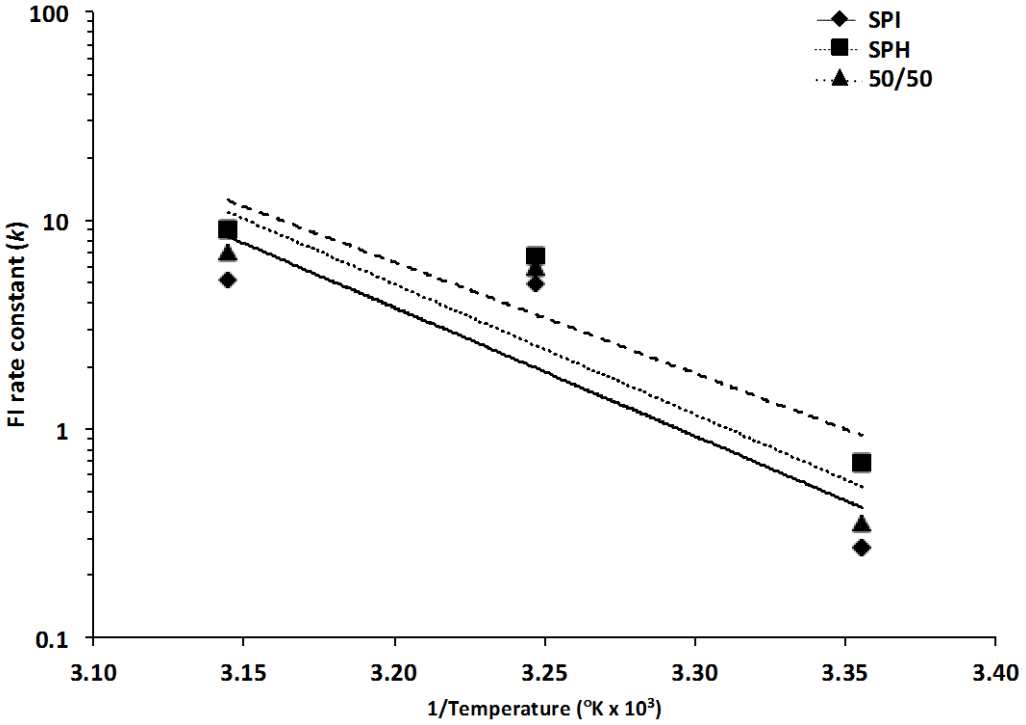

Figure 16. Arrhenius plot for the reaction rate constant (k) of fluorescence intensity (FI) of SPI, SPH, and 50/50 stored at 0.71-0.74 a_w (NaCl saturated salt solution) and 25, 35, and 45°C.

In this method, the excitation at 360 nm (bandwidth 40) and emission at 460 nm (bandwidth 30) were used because they were within the ranges of maxima for Maillard reaction fluorescent compounds: 340-370 nm for excitation and 420-475 nm for emission (Davies et al., 1998; Matiacevich et al., 2005; Morales et al., 1996; Sun & Leopold, 1995). These ranges are outside that of tryptophan, which fluoresces with an excitation of 290 nm and an emission of 335 nm (Matiacevich & Buera, 2006). It is also important to note that

the samples were deproteinized prior to %FI measurements; therefore, both free and previously protein-bound fluorescing compounds were detected. However, there is not just one compound that is identified at these excitation and emission values, so the identity of the compound(s) measured could not be determined.

Oxidized soybean flour had an excitation maximum at 360 nm, an emission maximum at 440 nm, and also followed pseudo-zero-order kinetics (Liang & Lin, 2000). Linolenic and linoleic fatty acids present in soybean flour could be released and oxidized by lipases and lipoxygenases, respectively, resulting in the formation of alcohols, ketones, and aldehydes. Lipid peroxidation products have been shown to stimulate Schiff base formation and generate fluorescence similar to that of Maillard reaction products (Castilho et al., 1994). However, the soybean flour utilized in our study was defatted with a reported fat % < 1.0%; and therefore, any remaining fatty acids would be present in a very small amount. In any case, there was a measureable amount of fluorescence in the original samples stored at -20°C and 0.05 a_w samples stored at 45°C. Therefore, it is possible that while the soy flour was stored at ambient temperature prior to making SPI and SPH, there could have been lipid oxidation products already forming. Stored soybeans have been shown to form lipid oxidation products (Narayan et al., 1988). It is probable that soybeans were also stored prior to soybean flour production.

Overall, the fluorescence results suggested that some Maillard reaction products were completing the intermediate stage and others were in the advanced stages in SPH, while those present in SPI and 50/50 were still entering the intermediate stage. This observation compliments the findings at 45°C and 0.79 a_w for the decrease in percent remaining free amino groups as SPH reached a plateau, while SPI and 50/50 continued losing and regenerating free amino groups (Figure 14). The change in percent remaining free amino group and increase in fluorescence indicated that the highest reactivity was at 0.79 a_w and not below. However, the exact reactants and products could not be determined. As reported by Davies *et al.* (1998), isoflavones may act as reactants for the formation of fluorescing and browning Maillard products. Having other reactants could also explain the constant change in reaction rate for the percent remaining free amino groups. More

research is necessary to determine the reactions that may be simultaneously occurring with the Maillard reaction to result in increased fluorescence.

2.5. Conclusions

Because the protein powders had similar intrinsic properties and moisture sorption isotherms, observed differences during storage were mostly attributed to their protein/peptide profiles. The SDS-PAGE gels revealed that aggregation and loss of solubility during storage of SPI, SPH, and 50/50 could be attributed to hydrophobic interactions and disulfide interchange. Soy protein isolate had the largest decrease in percent solubility as compared to SPH and 50/50, which meant it formed the most insoluble aggregates across a_w during storage at 45°C. The aggregation was not the result of protein denaturation but instead was attributed to increased protein molecular mobility and the formation of liquid bridges.

Although there were traces of glucose and other reducing sugars, the observed physicochemical changes indicated that the Maillard reaction did indeed occur in soy protein powders over time. Through tracking of color change (browning), loss of free amino groups, and the increase in fluorescence, the extent of the Maillard reaction was determined. The rate of fluorescence, loss of free amino groups, and change in color was highest above 0.70 a_w across all temperatures for all the protein powders. Overall, these measurements (fluorescence, loss of free amino groups, and color change) indicated that the extent of the Maillard reaction was greater in stored SPH than in SPI and 50/50.

During the storage studies at 25° and 35°C, there was only a small amount of change observed and only at 0.74 a_w. These findings implied that storage of dry soy protein ingredients below 0.74 a_w and near ambient temperature might not cause a rapid increase in aggregation, thus maintaining their original functional properties. Of course it is optimal to keep SPI and SPH at low temperatures (preferably 4°C), but the aggregation should be well controlled through keeping the a_w at or below the m_0. Additionally, formulation with soy protein ingredients should be kept below 0.70 a_w, or other ingredients such as humectants should be incorporated to prohibit texture changes due to moisture-induced

aggregation. It is recommended that soy protein powder manufacturers ensure that their storage and distribution follows these guidelines to minimize quality changes.

3. Changes in Nutritional Quality and Bioactivity in Soy Protein Powders During Accelerated Storage

3.1. Overview

With consumer interest in protein-enhanced foods as well as an increase in the utilization of protein hydrolysates, soy protein isolate (SPI) and hydrolysate (SPH) have become important protein ingredients due to their health benefits. Particularly the use of SPH is growing due to their physiological contributions, increased digestibility, enhanced functionality, and reduced allergenicity. Currently, no work has been done on the effect of storage on the antihypertensive activity of SPI and SPH as influenced by protein/peptide aggregation. This study examined the nutritional and physiological changes of SPI, SPH, and a 50/50 mixture of SPI and SPH during storage at 45°C under various water activities (a_w), ranging between 0.05 and 0.79 a_w. Although aggregation occurred during storage at high a_w, there was no significant change in protein digestibility and angiotensin-converting enzyme (ACE) inhibitory activity. However, loss in free amino groups was observed, due to the initiation of the Maillard reaction at 0.79 a_w for all sample types. The results indicated that protein aggregation induced during storage might not affect bioactivity or digestibility of soy protein powders, hence not limiting their application as functional food ingredients.

3.2. Introduction

Soy protein is considered a nutritious and healthy, vegetarian protein source. Containing over 90% protein, soy protein isolate (SPI) is a common food protein ingredient that is produced in mass quantities and shipped all over the world for utilization in nutrition/energy bars, cereals, meat alternatives, and more. Soy protein hydrolysate (SPH), which is produced through enzymatic hydrolysis of SPI, also has a high protein content and contributes many other benefits such as enhanced functionalities (Hettiarachchy & Kalapathy, 1998; Chiang et al., 1999; Martínez et al., 2009), physiological benefits attributed to bioactive peptides (Lovati et al., 1996; Martinez-Villaluenga et al., 2009; Margatan et al., 2013), and enhanced digestibility.

The consumption of soy protein has historically been of great concern because of the lectins and trypsin inhibitors naturally present in the soybean. Heat treatment can denature lectins and inactive trypsin inhibitors (Friedman & Gumbmann, 1986; Friedman & Brandon, 2001). However, heat treatment that results in protein denaturation has been shown to actually decrease the digestibility of legume proteins due to the formation of high molecular weight aggregates stabilized through disulfide linkages (Utsumi et al., 1984; Carbonaro et al., 2005). There is a lack of research on how storage affects digestibility of SPI and SPH. Dry protein powders are often stored for over a year or more and experience many changes in temperature and relative humidity (RH). It is necessary to characterize how the aggregation that can occur with adverse temperature and RH affects the nutritional benefits, including digestibility, of these protein ingredients.

Depending on the hydrolysis conditions employed, SPH may be a good source of bioactive peptides. Bioactive peptides are short amino acid sequences that upon release from the parent protein may contribute to several physiological benefits including antihypertensive, cancer-preventative, and antioxidant activity (Hoppe et al., 1997; Park et al., 2007; Margatan et al., 2013). Among the most researched bioactive peptides in SPH, are the ones that inhibit the angiotensin-converting enzyme (ACE) (Wu & Ding, 2001; Wu & Ding, 2002; Gibbs et al., 2004; Margatan et al., 2013). Angiotensin-converting enzyme raises blood pressure by catalyzing the conversion of inactive angiotensin I to active angiotensin II, a vasoconstrictor hormone in the renin angiotensin system (RAS); also,

ACE degrades bradykinin, a vasodilator (Skeggs et al., 1954, 1956; Grafe et al., 1993). By inhibiting ACE activity, the RAS is better controlled and may result in the prevention of hypertension. Hypertension, which affects over 29% of U.S. adults aged 18 and over, is one of the main risk factors for cardiovascular diseases (Nwankwo et al., 2013). The prevalence of hypertension significantly increases as people age, with 65% aged 60 and older diagnosed as hypertensive (Nwankwo et al., 2013). Therefore, it is important to control hypertension especially as the elderly population continues to grow in the United States.

With the increased use of hydrolysates, come the challenges of controlling the changes that can occur during the adverse conditions of processing and storage. Thermal treatment during processing and storage can cause proteins to aggregate, causing losses in solubility (Mori et al., 1982; LaClair & Etzel, 2009), and decreases in nutritional quality (Adrian & Frangne, 1973; Desrosiers et al., 1989; O'Brien & Morrissey, 1989). Margatan *et al*. (2013) has shown that heat treatments of 120° and 175°C on SPH produced through papain hydrolysis—which selectively hydrolyzes β-conglycinin—does not significantly affect SPH's ACE inhibitory activity. In addition, Wu & Ding (2002) have shown that soy-derived ACE inhibitory peptides were resistant to *in vitro* digestion, heat treatments up to 100°C, and 2.0-10.0 pH treatments. However, the stability of soy-derived peptides' ACE inhibitory activity when subjected to adverse storage temperatures and relative humidities (RH) has not been investigated thus far.

Although several studies have investigated the digestibility and ACE inhibitory activity of soy proteins/peptides, studies of the effect of storage conditions on the nutritional and physiological benefits of SPH are lacking. Without knowing how the health benefits of soy proteins powders are effected during processing and storage, they cannot be fully utilized as health-enhancing food ingredients. Therefore, the main purpose of this study was to examine changes of the ACE inhibitory activity of SPH as well as the digestibility of SPI and SPH after storage at 45°C and various RH. The nutritional and physiological properties as affected by aggregation and the Maillard reaction that occurred at these adverse conditions—as was shown in the previous chapter—were thus investigated.

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 Angiotensin Converting Enzyme from rabbit lung (2.0 units/mg), N-[3-(2-Furyl)acryloyl]-Phe-Gly-Gly (FAPGG), pepsin from porcine gastric mucosa (3,200-4,500 units/mg), and trypsin type 1X-S from porcine pancreas (10,000 BAEE units/mg) purchased from Sigma Aldrich (St. Louis, MO, USA).

3.3.2. Preparation of SPI

Soy protein isolate (~90% protein) was prepared in the same manner as outlined in Section 2.3.2.

3.3.3. Preparation of SPH

Soy protein hydrolysate (~90% protein, ~6% degree of hydrolysis) was prepared in the same manner as outlined in Section 2.3.3.

3.3.4. Storage Study Experimental Design

Soy protein isolate, SPH, and a 50/50 mixture of SPI and SPH (from herein referred to as 50/50) were stored in the same manner as described in Section 2.3.4. However, only samples stored at select a_w (0.05, 0.59, 0.71, and 0.79 a_w) at 45°C were assessed for changes in digestibility and ACE inhibitory activity as the changes (color change, increase in aggregation, loss of free amino groups, decrease in solubility, and increase in fluorescence intensity) observed at these storage conditions were more drastic than those at the other temperatures.

3.3.5. Loss of Free Amino Groups

The loss of free amino groups was determined by the o-phthaldialdehyde (OPA) method as outlined in Section 2.3.13.

3.3.6. Measurement of Angiotensin-Converting Enzyme (ACE) Inhibitory Activity

The ACE inhibitory activities of SPH and 50/50 were measured, in triplicate, following the assay outlined by Otte *et al.* (2007) and Shalaby *et al.* (2006), and modified by Margatan *et al.* (2013), using furanacryloyl-L-phenylalanylglycylglycine (FA-PGG) and sodium borate buffer (0.1 M sodium tetraborate with 0.3 M sodium chloride, pH 8.3 at 37°C). The ACE was dissolved in cold distilled, deionized water (DDW) to reach a final concentration of 0.288 units/mL and then aliquoted and frozen at -20°C until usage. The FA-PGG was prepared in 0.1 M sodium borate buffer to a concentration of 0.88 mM and subsequently aliquoted and frozen at -20°C until usage. Soy protein hydrolysate samples were prepared by diluting samples (20 mg/mL) in DDW, centrifuging at 15,682 x g for 10 minutes, and then filtering through 0.45 μm syringe filters. The filtered solutions were further diluted to concentrations of 7.5, 10.0, 12.5, 15.0, and 17.5 mg solids/mL. Protein concentration of the supernatant was determined using the Thermo Scientific™ Pierce™ BCA assay kit, following manufacturer instructions. The reaction mixtures (as outlined in Table 10) were prepared in a 96-well plate and their absorbance was read at 340 nm every 30 seconds for 30 minutes while in the microplate reader (BioTek Synergy HT, BioTek Instruments, Winooski, VT, USA) at 37°C.

The ACE inhibitory activity was expressed as the slope of the decrease in absorbance at 340 nm taken from 10-25 minutes of incubation. The IC_{50} values (inhibitor concentrations that inhibit ACE by 50% in comparison to the control) were determined by plotting % ACE inhibition by protein concentration. The % ACE inhibition by sample solutions was calculated using **Equation 9** and **Equation 10**. The IC_{50} value was deduced from the plot of % of ACE activity reduction vs. the concentration of SPH using the equation of the line (**Equation 11**).

Equation 9:

$$\text{Conc. of protein in the well } (\text{mg/mL}) = \frac{\text{sample concentration } (\text{mg/mL}) * 10\mu\text{L}}{170\mu\text{L}}$$

Equation 10:

$$\%\text{ACE Inhibition} = \frac{\text{slope}_{\text{control}} - \text{slope}_{\text{inhibitor}}}{\text{slope}_{\text{control}}} * 100\%$$

Equation 11:

$$IC_{50} = y = mx + b$$

Table 10. Reaction mixture for ACE activity assay.

	Na-borate buffer[a] (μL)	FA-PGG[b] (μL)	Sample[c] (μL)	ACE[d] (μL)
Control blank	170	—	—	—
Control	10	150	—	10
Sample blank	170	—	—	—
Sample	—	150	10	10

[a] Sodium tetraborate buffer: 0.1 M borate, 0.3 M chlorine ion, pH 8.3
[b] 0.88 nM FA-PGG in Na-borate buffer
[c] Dilutions of either SPH or 50/50 in DDW
[d] 0.288 units/mL ACE in DDW

3.3.7. Change in Protein Digestibility

Digestibility of SPI, SPH, and 50/50 was determined, in duplicate, following the sequential *in vitro* digestion by pepsin and trypsin as outlined by Tang *et al.* (2006), with modifications. Dispersions (5 mL of 1% protein, w/v) of soy protein samples were prepared in 0.1 N HCl at a pH of 1.5 and warmed up to 37°C in a water bath with gentle stirring. After 10 minutes of incubation, pepsin (100 μL of 3 mg/mL) was added to the samples and incubated at 37°C with constant stirring for 2 hours. Then, the pH was adjusted to 7.0 using 1.0 N NaOH, to terminate pepsin activity, and trypsin (100 μL of 9 mg/mL) was added to each of the samples and incubated for 2 hours at 37°C. The pH was periodically checked and adjusted during incubation to keep it at 7.0. After the digestion, the samples were removed from the water bath and 1 mL of 1% SDS and 5% β-mercaptoethanol was added to enhance solubility of all proteins present. Samples were then filtered through 0.45 μm filters and immediately frozen to -20°C for further analysis.

The peptide profiles of the samples were analyzed via high performance liquid chromatography (HPLC) based on the method outlined by Chevalier *et al.* (2001), with modifications. An HPLC system (Shimadzu Corp., Kyoto, Japan) equipped with a LC-

20AT, CTO-20A oven, and SPD-20A UV/Vis 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. A linear binary gradient was employed using HPLC grade water (solvent A) and acetonitrile (solvent B), with both containing 0.11% (v/v) trifluoroacetic acid. After injecting 50 μL of sample, solvent B was linearly increased from 16% to 40% in 20 minutes, kept constant for 10 minutes, increased to 80% for 10 minutes, decreased to 16% in 10 minutes, and then followed by column equilibration steps. 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 and proteins were monitored at 214 and 280 nm, respectively. The percent digestibility was determined based on the differences in peak areas obtained at 214 nm, which corresponded to peptide bonds. To calculate % digestibility, **Equation 12** was used (example calculation can be found in Appendix K).

Equation 12:

$$\%\ \text{Digestibility} = \frac{\text{peak area}_{\ \text{non-digested}} - \text{peak area}_{\ \text{digest}}}{\text{peak area}_{\ \text{non-digested}}} * 100\%$$

3.3.8. 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 L (Tables 20 & 21).

3.4. Results and Discussion

3.4.1. Effects of Storage on the ACE Inhibitory Activity of SPH and 50/50

The ACE inhibitory activity prior to storage for SPH and 50/50 was determined in order to assess changes resulting from storage at the a_w levels that caused aggregation, decreased solubility, and indications of the Maillard reaction. The original 50/50 sample

had lower ACE inhibitory activity than SPH because it had less peptides available per gram protein. A regression line with a steep slope indicated high enzyme activity, and thus a reduced slope indicated an inhibition of the enzyme activity (Figure 17). When diluted to the same concentration, 50/50 had a slope over 1.5 times greater than SPH; however, both 50/50 and SPH had considerably lower slopes than the control (ACE with no inhibitor added), thus confirming ACE inhibitory activity. The differences were made even clearer through determining the IC_{50} values (Figure 18) because of the calculations made to correct for solubilized protein. A lower IC_{50} value corresponded to higher ACE inhibitory activity because less protein was required to cause a 50% inhibition.

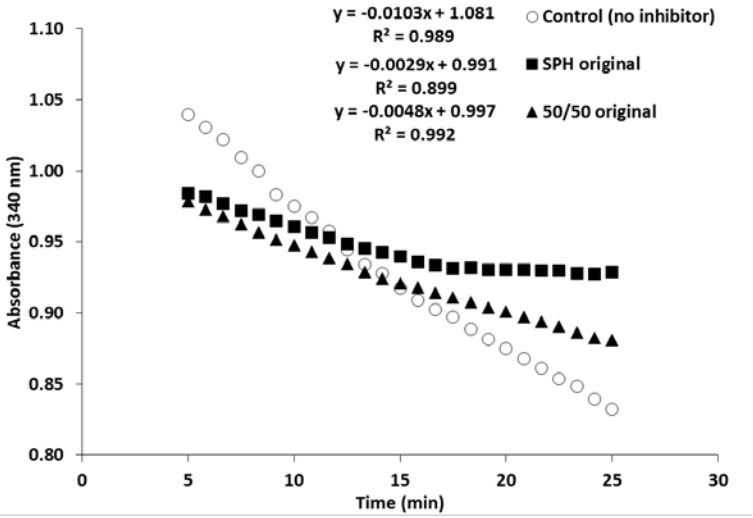

Figure 17. ACE inhibitory activity of Control (FA-PAGG and ACE, no inhibitor), SPH original (15 mg solids/mL), and 50/50 original (15 mg solids/mL). The lower the slope, the higher is the enzyme inhibitory activity.

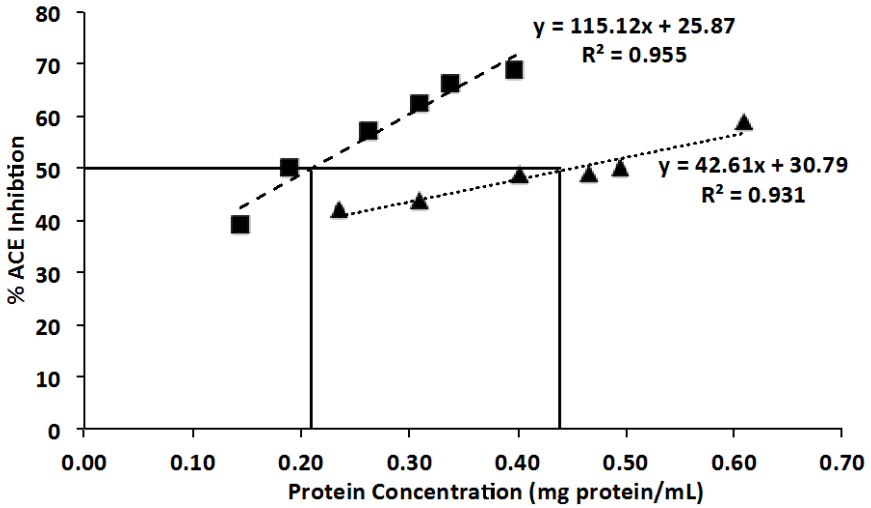

Figure 18. Example of dose-dependent inactivation of ACE by original SPH and 50/50 samples. Equations represent those used to calculate the IC_{50} with y = 50.

The original SPH sample had an average IC_{50} of 0.259 ± 0.026 mg protein/mL, which fell within the range of IC_{50} values—0.046-0.930 mg protein/mL—that were observed in literature for soy-derived peptides (Cha & Park, 2005; Chiang et al., 2006; Lo & Li-Chan, 2005; Margatan et al., 2013). It is important to note that the majority of research with low IC_{50} values reported for soy-derived peptides was produced through extensive hydrolysis followed by separation and purification steps. Although there was no significant ($P \leq 0.05$) difference between the IC_{50} value of the original SPH sample and the stored samples, there was a significant ($P \leq 0.05$) difference between the samples stored at 0.05 a_w and all the other high water activities tested (Figure 19). Because greater amounts of smaller peptides have been shown to increase the overall bioactivity (Wu & Ding, 2002), it is possible that the presence of endogenous or microbial proteases resulted in further hydrolysis during storage at high a_w. It was hypothesized that at low a_w of 0.05, these proteases would not be active or have enough mobility to continue cleaving peptide bonds during storage, and therefore the ACE inhibition remained the same.

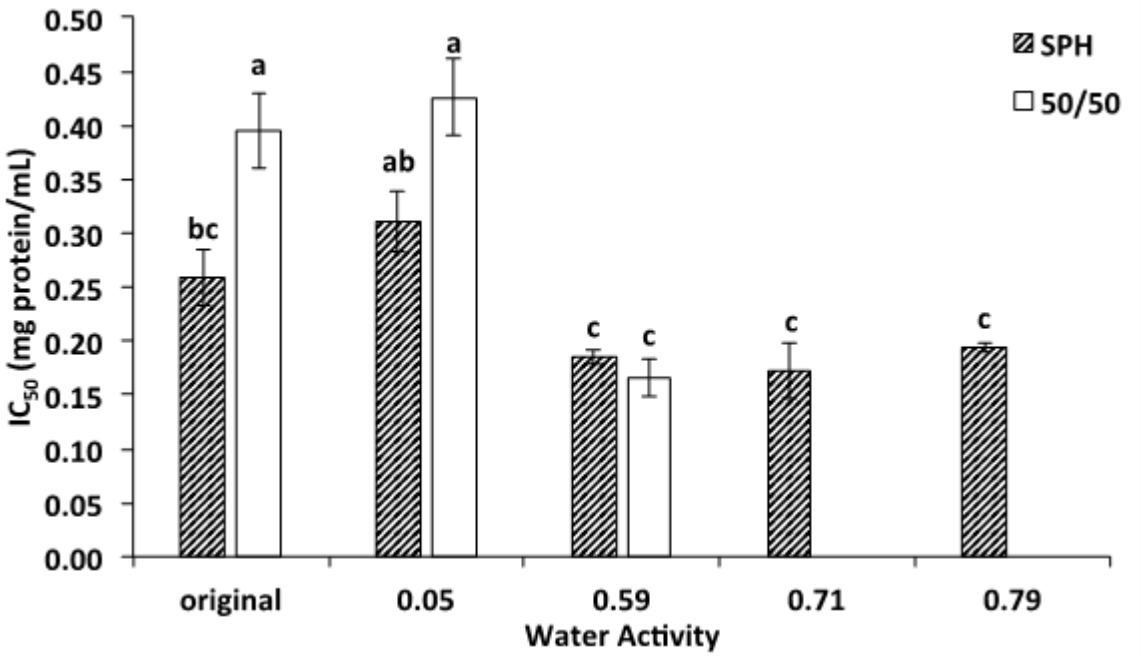

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Figure 19. Effect of a_w on ACE inhibitory activity of SPH and 50/50 after 77 days of storage at 45°C. Error bars represent standard error (n=3). Lowercase letters above the bars indicate significant difference between the means as determined following the Tukey-Kramer multiple means comparison test ($P \leq 0.05$).

Because of the unexpected trend of lower IC_{50} values for samples stored at higher a_w, the IC_{50} values of 50/50 for select samples were determined to test the hypothesis that further hydrolysis of proteins was occurring. The original (not stored) 50/50 sample had an IC_{50} of 0.395 ± 0.034 mg protein/mL. A similar trend for 50/50 was observed compared to that of SPH (Figure 19). The IC_{50} value for 50/50 at 0.79 a_w was significantly ($P \leq 0.05$) lower than that of the original sample, which supported the hypothesis of endogenous and/or microbial proteases present. As discussed in Chapter 2, a large extent of insoluble aggregate formation was noted for 50/50 after storage at 0.79 a_w and 45°C for 77 days and some aggregation was also observed for SPH at the same parameters. According to the ACE inhibitory results, the bioactive peptides did not seem to be involved in the aggregate formation, and thus remained bioactive. However, future *in vivo* studies and storage research conducted using with antimicrobial agents would help support this theory.

3.4.2. Effect of Storage on the *in vitro* Digestibility of SPI, SPH, and 50/50

To analyze whether the aggregation and loss of solubility had an effect on nutritional quality, *in vitro* digestibility was assessed for the samples that experienced high amounts of aggregation and was compared to that of the original samples and those stored at 0.05 a_w. The chromatographic peaks that changed the most after digestion had retention times between 37 and 44 minutes (Figure 20). The areas of these peaks pre and post digestion were used to calculate the percent digestibility. Because a C-18 column was used, these peaks were hypothesized to be more hydrophobic and larger in molecular weight, in contrast to the more hydrophilic peptides represented by the chromatographic peaks with retention times ranging from 0 to 20 minutes (Figure 20).

Depending on the soy protein treatment and analysis, a range of protein digestibility (60-97%) has been reported in literature (Friedman & Brandon, 2001; Tang et al., 2006; Hughes et al., 2011; Li et al., 2013). In this study, prior to storage, SPI, SPH, and 50/50 had 73%, 83%, and 77% digestibility, respectively. Across all sample types and a_w, there was no significant ($P \leq 0.05$) difference between the original samples and their stored counterparts (Figure 21). However, the % digestibility of SPI stored at 0.59 a_w was significantly ($P \leq 0.05$) higher than SPI stored at 0.05 a_w. Soy protein isolate experienced a drastic decrease in solubility at 0.59 a_w, while there was no difference in solubility for SPI stored at 0.05 a_w (Figure 10, A). These findings suggested that an increase in insoluble aggregate formation does not necessarily indicate a decrease in digestibility. Yet, the % digestibility of SPI stored at 0.59 a_w was also significantly ($P \leq 0.05$) higher than SPI stored at 0.79 a_w, which suggested that extensive aggregation resulted in larger polymers that have decreased digestibility. Soy protein isolate stored at 0.79 a_w and 45°C for 77 days was the only sample that displayed high molecular weight aggregates formed through covalent linkages (S3 fraction, Figure 13, A), making it the sample with the largest extent of aggregation. Interestingly, the % digestibility at 0.79 a_w was not significantly ($P \leq 0.05$) different from that of the original sample. This observation could be attributed to the presence of proteases that might have resulted in protein hydrolysis, counteracting the effect of large polymer formation on digestibility.

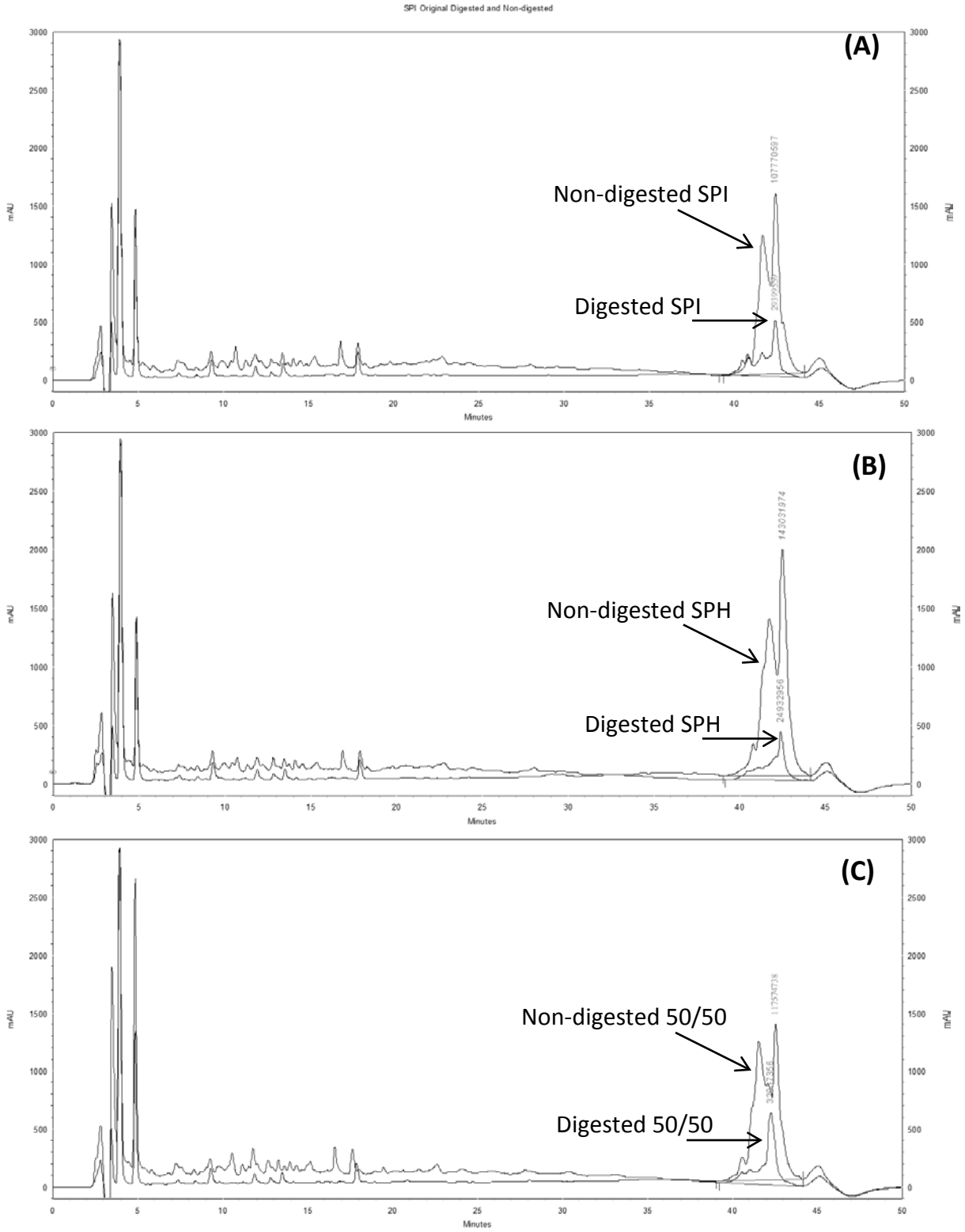

Figure 20. Chromatograms (at 214 nm) of original non-digested and digested SPI (A), SPH (B), and 50/50 (C). Peak areas indicated.

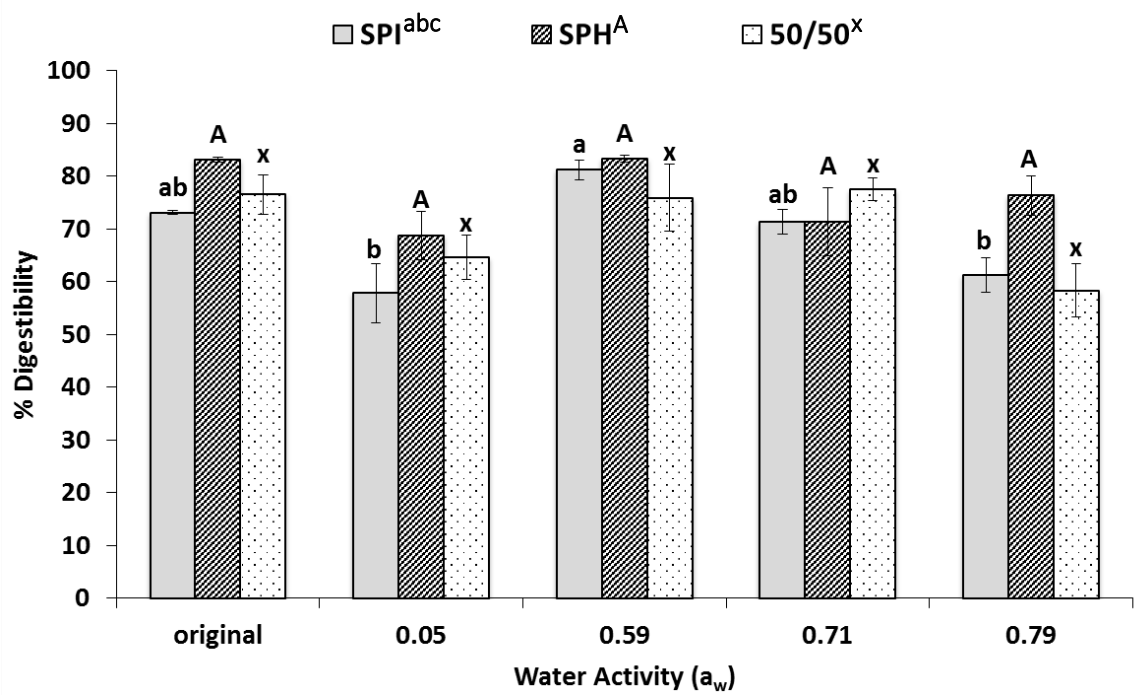

Figure 21. Effect of a_w on digestibility of SPI, SPH, and 50/50 after 77 days of storage at 45°C. Error bars represent standard error (n=3). Lowercase letters above the bars indicate significant difference (abc for SPI, A for SPH, and x for 50/50) between the means as determined following the Tukey-Kramer multiple means comparison test ($P \leq 0.05$).

As mentioned previously, microbial and/or endogenous proteases may have been present during storage. Therefore, the difference between the percent digestibility at 0.05 and 0.59 a_w may have been due to some hydrolysis that already occurred throughout storage. This hypothesis was supported by the findings of % remaining free amino groups at 0.59 a_w and 45°C, where the % remaining free amino group significantly ($P \leq 0.05$) exceeded that of the original sample after 40 days of storage (Figure 29, A, Appendix H). Overall, the retention of protein digestibility during adverse storage conditions was consistent with other findings in literature. Research by da Silva Pinto *et al.* (2005) showed no significant change for *in vitro* digestibility when storing SPI and SPH at 42°C for 1 year even though there was a significant loss in solubility. Although at a much lower storage temperature, Sathe *et al.* (2009) also showed no change for *in vitro* digestibility when storing defatted soy flour at -20°C for 20 years. However, storage of protein that had been denatured might have resulted in the formation of large polymers across more a_w, which

might be less digestible than the aggregates formed during this storage study. Results from the previous chapter showed that SPI was not denatured (Figures 33 & 34, Appendix I), thus very large polymers did not form. This may explain why a reduction in digestibility was not observed in comparison to the original samples' % digestibility. Future work that takes into account the proteins' denaturation state would further confirm this hypothesis.

3.4.3. Effect of Loss of Free Amino Groups on Nutritional Quality

One way to measure the retention of nutritional quality after different treatments is to assess the degradation of essential amino acids. This has been regularly examined in milk and cereal proteins associated with infant formula, but rarely with soy protein (Vigo et al., 1992; Ramírez-Jiménez et al., 2004; Bosch et al., 2007). In the past decade, soy protein has comprised over 25% of infant formula (Mendez et al., 2002; Bhatia & Greer, 2008) yet still, only limited work has been done to assess its nutritional contributions after storage and non-enzymatic browning.

During storage at 45°C, there was an overall loss of free amino groups at 0.79 a_w (Figure 10) and no loss at 0.05 a_w (Figure 30, Appendix H). Free amino group loss was primarily attributed to the Maillard reaction, which had a relatively high prevalence in SPI, SPH, and 50/50 at 0.79 a_w. After 77 days at 0.79 a_w, SPI, SPH, and 50/50 had 74.3 ± 4.9%, 82.3 ± 1.3%, and 79.3 ± 4.4% free amino groups remaining, respectively. Inconsistent trends were observed for sample types at other a_w. The inconsistent trends were attributed to endogenous and/or microbial proteases present that could cleave peptide bonds while the Maillard reaction was simultaneously occurring. After 77 days of storage at 0.71 a_w, SPH and 50/50 had significantly ($P \leq 0.05$) less free amino groups remaining at 78.5 ± 3.8% and 86.8 ± 1.4%, respectively, while SPI retained its free amino groups (Figure 28, Appendix H). At 0.59 a_w, SPI free amino groups increased above the starting value while SPH and 50/50 again experienced some losses and regains of free amino groups throughout storage (Figure 29, Appendix H).

The nutritional implications for the loss of free amino groups have been primarily researched in regards to the loss of lysine, an essential amino acid. That is because the ε-amino group in lysine is the most reactive free amine in the Maillard reaction. Other amino

groups such as terminal amines and the guanidinium group of arginine (essential for infants and children) and imidazole group of histidine (also an essential amino acid) can be involved in the Maillard reaction (Ajandouz & Puigserver, 1999). A decrease in percent of essential amino acids due to the Maillard reaction has negative effects on the overall nutritional quality. This is especially important for infant formula and when the food is touted for its nutritional benefits in energy/nutrition bars and beverages. Further research to determine the specific amino acids that were affected the most would help determine the extent of nutritional loss.

3.5. Conclusions

Storage at various a_w levels at 45°C, for an extended period of time, did not have a considerable effect on digestibility or ACE inhibitory activity of the different protein powders, regardless of their loss in solubility and aggregate formation. Because peptides produced during hydrolysis did not seem affected by aggregation, the functional and health benefits associated with SPH may remain stable even during long-term adverse storage conditions. On the other hand, the Maillard reaction that was initiated during storage of SPI, SPH, and 50/50 at high a_w resulted in a moderate loss of free amino groups, suggesting a potential loss of lysine, and thus nutritional quality. More research is necessary to determine the exact amino acids affected and if their loss has a significant effect on nutritional quality *in vivo*.

4. Overall Conclusions, Implications, and Recommendations

This work provides basic information for soy protein ingredients in terms of predicting and controlling storage stability. Physicochemical characteristics of SPI, SPH, and 50/50 were most affected at 45°C and high a_w. Because storage at 25 and 35°C at the tested a_w did not induce drastic changes, soy protein powders might not aggregate rapidly when kept at ambient temperatures and a_w<0.70. Although it is commonly recommended to store protein powders in refrigerated conditions to avoid any changes, it would be wiser to invest in packaging that highly controls water uptake.

The findings of this study also highlighted the importance of controlling storage conditions at all steps of the protein powder's production and distribution. Lipid oxidation and the Maillard reaction can occur in stored soybeans as well as soy flour, triggering reactions that will continue throughout the shelf life of protein powders and the final product. Protein powder manufacturers and future protein aggregation studies—especially those utilizing legume and/or seeds as resources—should highly consider tracking the initial resources' storage parameters. Simply tracking intrinsic properties may not properly predict future aggregation, the storage history is also important. As this study showed, minimal amounts of reducing sugars can still influence detrimental reactions in dry powder systems.

This study demonstrated, for the first time, that soy protein-derived peptides with ACE inhibitory activity retain their bioactivity throughout adverse storage conditions. However, this observation is highly dependent on the enzyme used and the resulting peptides. Also, this study demonstrated that extensive hydrolysis and purification is not necessary to produce peptides that have high ACE inhibitory activity. Based on our findings, when incorporated into a food system with any a_w, low or high, the initial bioactivity of the hydrolysate produced in this study will be maintained throughout storage. To be able to incorporate soy protein ingredients with bioactivity into functional foods, further studies utilizing intermediate moisture food (IMF) model systems should be completed. Also, *in vivo* studies to determine ACE inhibitory activity would help prove this hypothesis.

As a relatively cheap and sustainable resource, soy protein should be utilized to its full extent as a nutritional and functional food. Continued research on soy protein should incorporate the ingredients currently utilized in the food industry, such as those that are optimized for enhanced solubility in nutritional beverages. Because there was a difference in heat treatments prior to storage of the different powders, it is recommended that research further assess the influence of denaturation state in SPI and SPH on aggregation. Based on our findings, non-denatured SPI and denatured SPH should maintain their % digestibility through storage. However, because non-denatured proteins are less likely to form large polymers, the use of an immobilized enzymatic system to produce SPH could also be explored to avoid the boiling step performed in this work. The recommended future studies will provide a wider understanding on how proteins aggregate in more complex systems, leading to solutions for maintaining optimum quality during storage.

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Appendix A: Calculation for Moisture Content Determination

Equation 13

$$\mathrm{MF} = \frac{\text{MeOH mass (g)}}{\text{Injection mass (g)}}$$

Equation 14

$$\text{Corrected } \mathrm{H_2O} \text{ (mg)} = \left(\frac{\mathrm{R}}{1000} * \mathrm{MF}\right) - \text{MeOH mass } * \text{Total } \mathrm{H_2O_{blank}}$$

Equation 15

$$\%\mathrm{H_2O_{\mathbf{wb}}} = \frac{\text{Corrected } \mathrm{H_2O} \text{ (mg)}}{10 * \text{Sample mass (g)}}$$

Equation 16

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

Where:

MeOH = methanol

MF = multifactor

R = water (ug)

Appendix B: Example Standard Curves

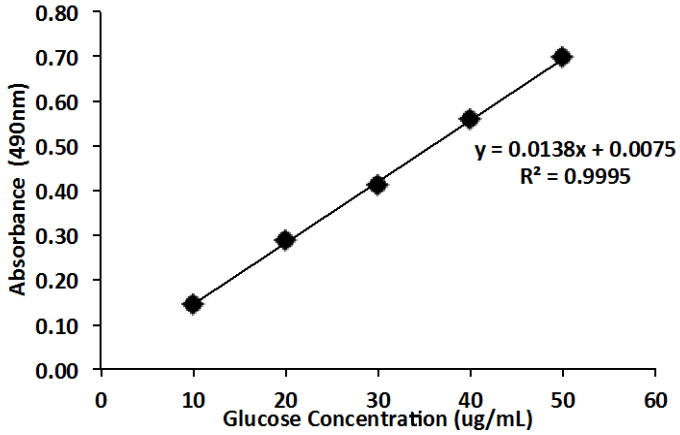

Figure 22. Total carbohydrate glucose standard curve.

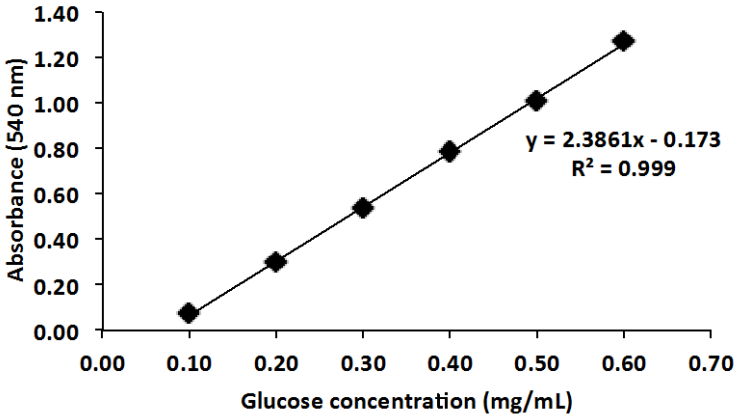

Figure 23. DNS assay glucose standard curve.

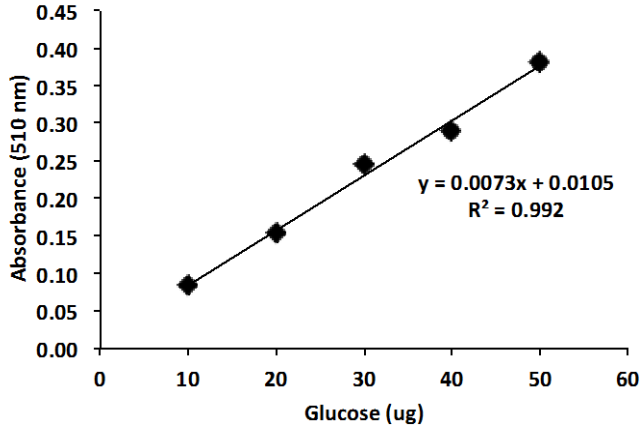

Figure 24. Enzymatic assay glucose standard curve.

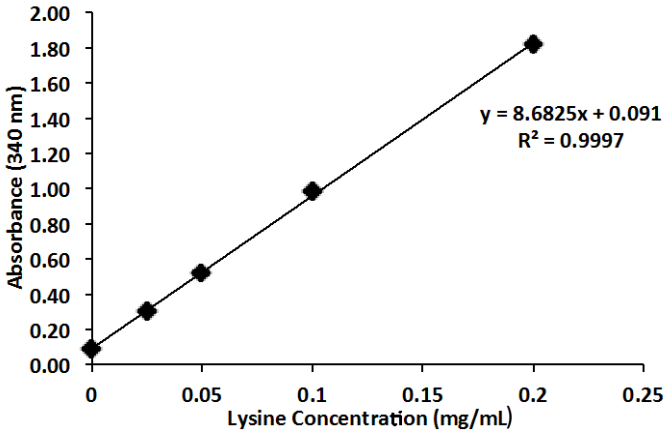

Figure 25. Free amino groups lysine standard curve.

Appendix C: Example Calculation for Degree of Hydrolysis (DH)

Example calculation for DH of SPH.

Equation 1

$$\text{Serine} - \text{NH}_2 = \frac{0.3631 - 0.0631}{0.7731 - 0.0631} * 0.9516 \frac{\text{meqv}}{\text{L}} * 0.01 * \frac{100}{0.010 * 38.36}$$

$$\text{Serine} - \text{NH}_2 = 1.05$$

Equation 2

$$\text{h} = \frac{(0.50 - 0.342)}{0.97}$$

$$\text{h} = 0.73$$

Equation 3

$$\text{DH} = \frac{0.73}{7.8} * 100$$

$$\text{DH} = 9.33$$

Equation 4

$$\text{Corrected DH} = 9.33 - 3.47$$

$$\text{Corrected DH} = 6.56$$

Appendix D: GAB Isotherm Equations

GAB Isotherm Equations

The GAB graph was created by plotting a_w/moisture content on the y-axis and a_w on the x-axis as shown by the GAB equation (**Equation 17**). The y-intercept and slope of the 2nd polynomial trend line were used to calculate the m_0 (**Equation 18**) and C (**Equation 19**), and k (**Equation 20**).

Equation 17

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

Equation 18

$$m_0 = \frac{1}{\alpha \times k \times C}$$

Equation 19

$$C = 2 + \frac{\beta}{\alpha \times k}$$

Equation 20

$$k = \frac{\sqrt{\beta^2 - 4\alpha\varepsilon} - \beta}{2 \times \varepsilon}$$

Where:

a_w = water activity

m_0 = monolayer moisture value

k = multilayer constant

C = surface heat constant

Appendix E: Example Calculation for Total Color Difference (ΔE)

Example calculation for ΔE of SPH after 84 days of storage at 45°C and 0.79 a_w.

Equation 5

$$\Delta E = \sqrt{(81.66 - 87.86)^2 + (4.67 - -0.86)^2 + (25.37 - 19.16)^2}$$

$$\Delta E = 10.37$$

Appendix F: ESI-MS Spectra for Sugars in SPH

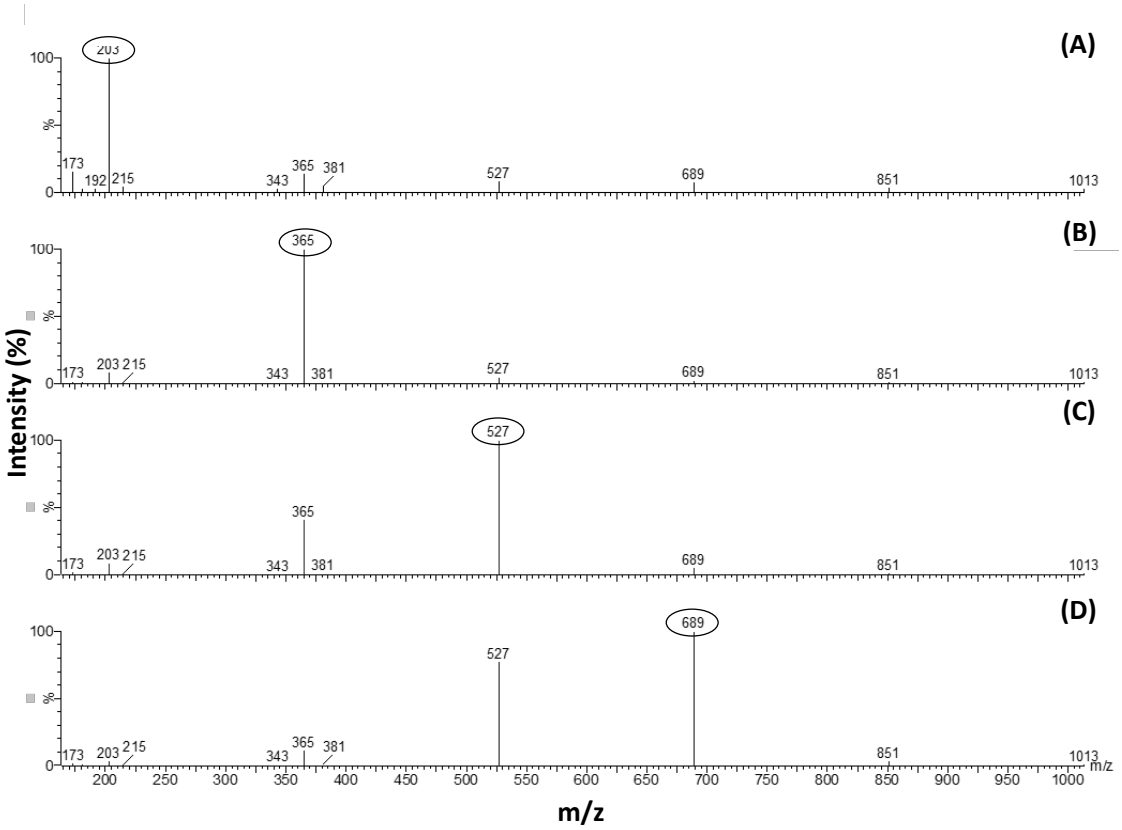

Figure 26. ESI-MS analysis of the sodium adducts of the sugars extracted for SPH: glucose (A), sucrose (B), raffinose (C), stachyose (D). Circled ions represent the indicated sugar for each spectrum.

Appendix G: Color Change at 25° and 35°C

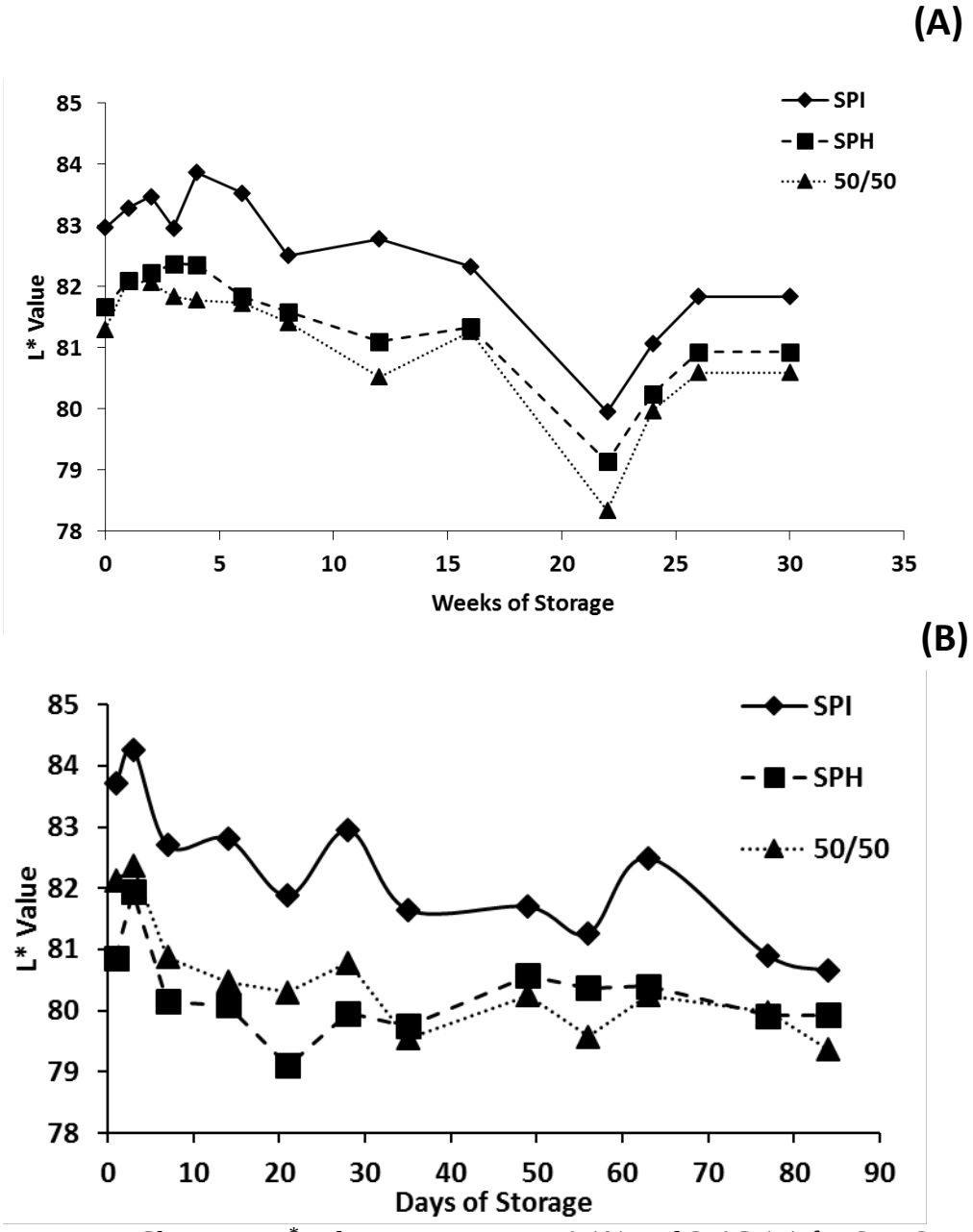

Figure 27. Change in L^* value over time at 25° (A) and 35°C (B) for SPI, SPH, and 50/50.

Appendix H: Change in Percent of Free Amino Groups

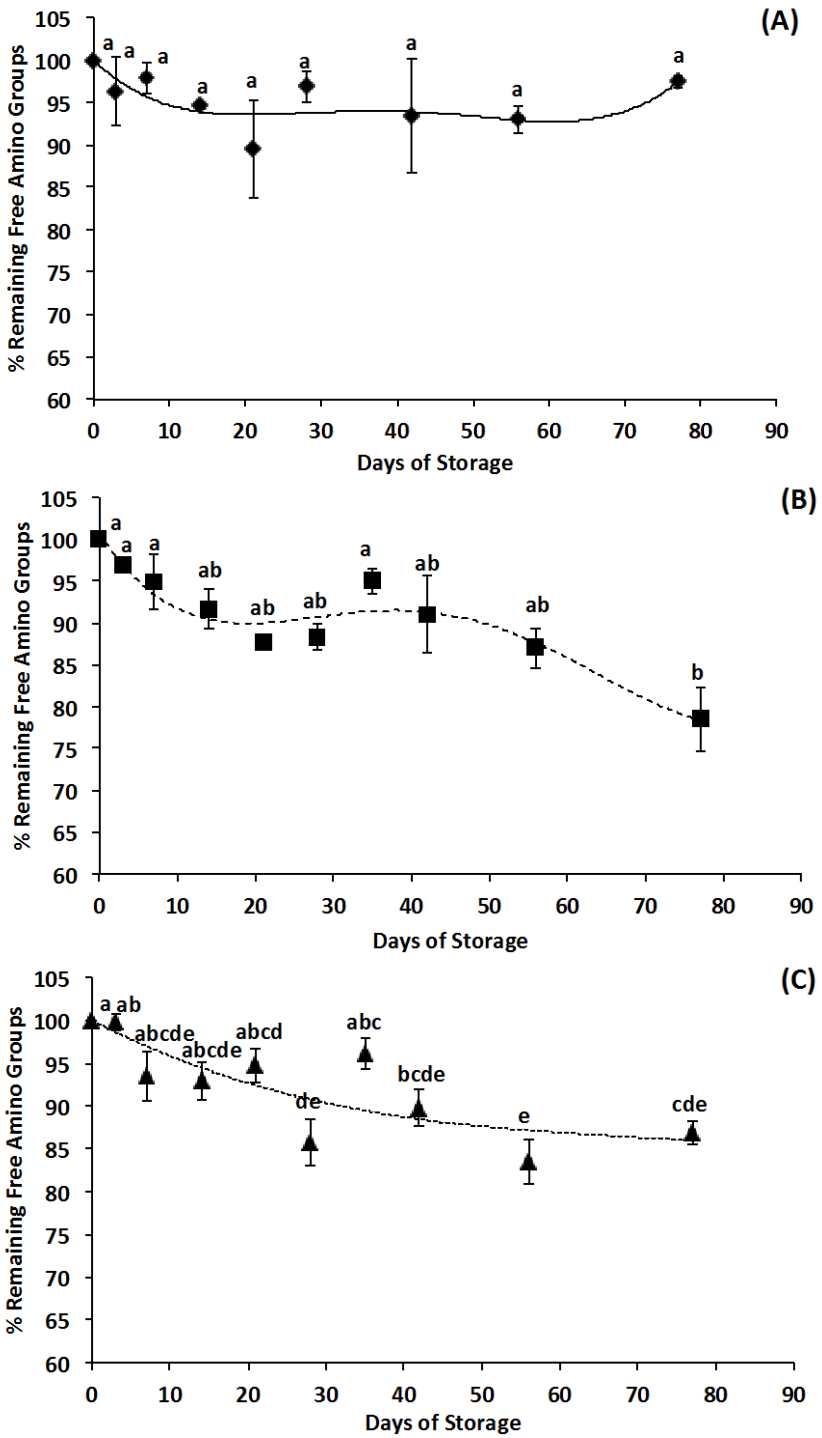

Figure 28. Change in % remaining free amino groups in SPI (A), SPH (B), and 50/50 (C) during storage at 0.71 a_w and 45°C. Error bars represent standard error (n=3). Lowercase letters above each point indicate significant difference between the means as determine following the Tukey-Kramer multiple means comparison test ($P \leq 0.05$).

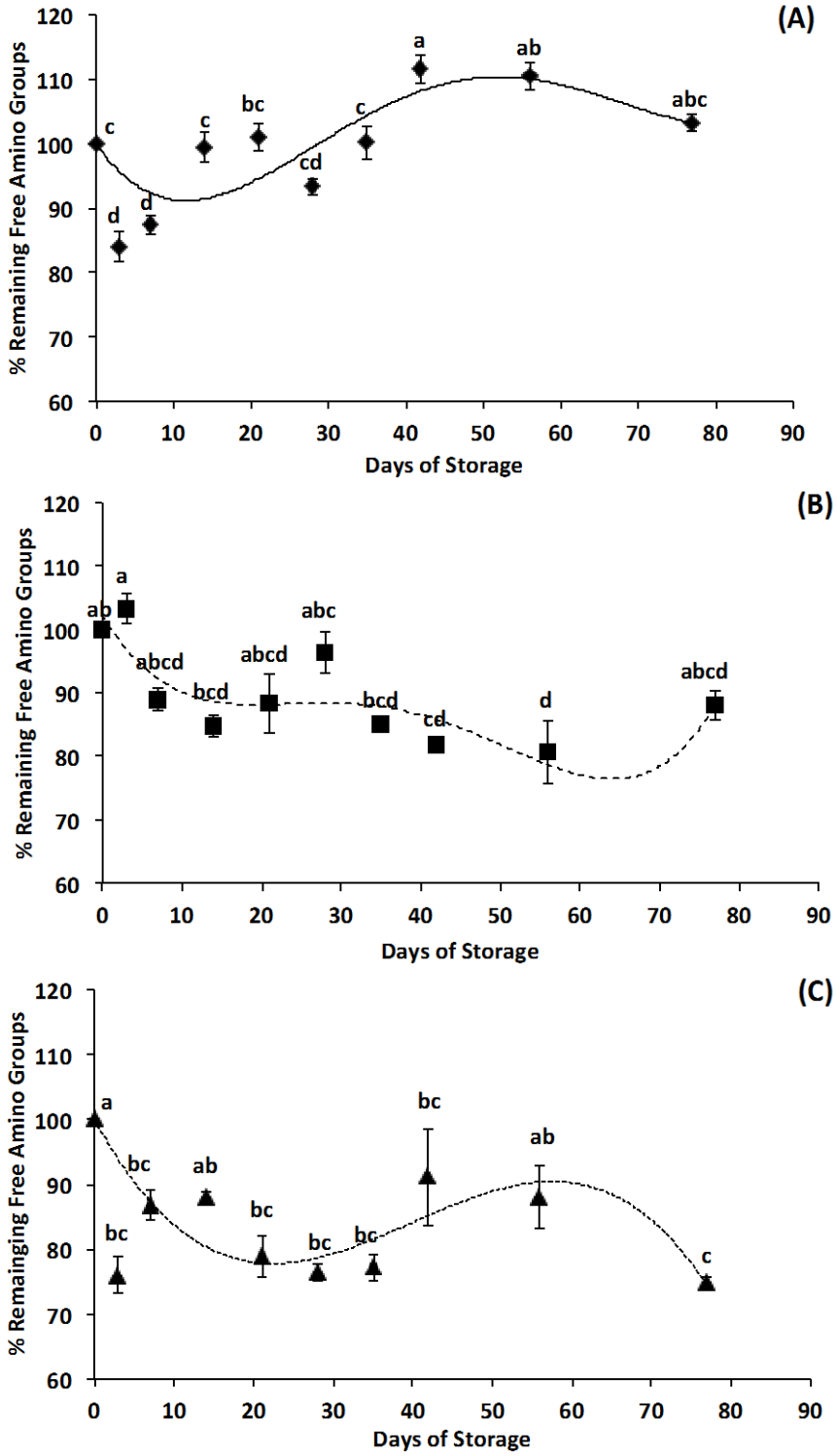

Figure 29. Change in % remaining free amino groups in SPI (A), SPH (B), and 50/50 (C) during storage at 0.59 a_w and 45°C. Error bars represent standard error (n=3). Lowercase letters above each point indicate significant difference between the means as determine following the Tukey-Kramer multiple means comparison test ($P \leq 0.05$).

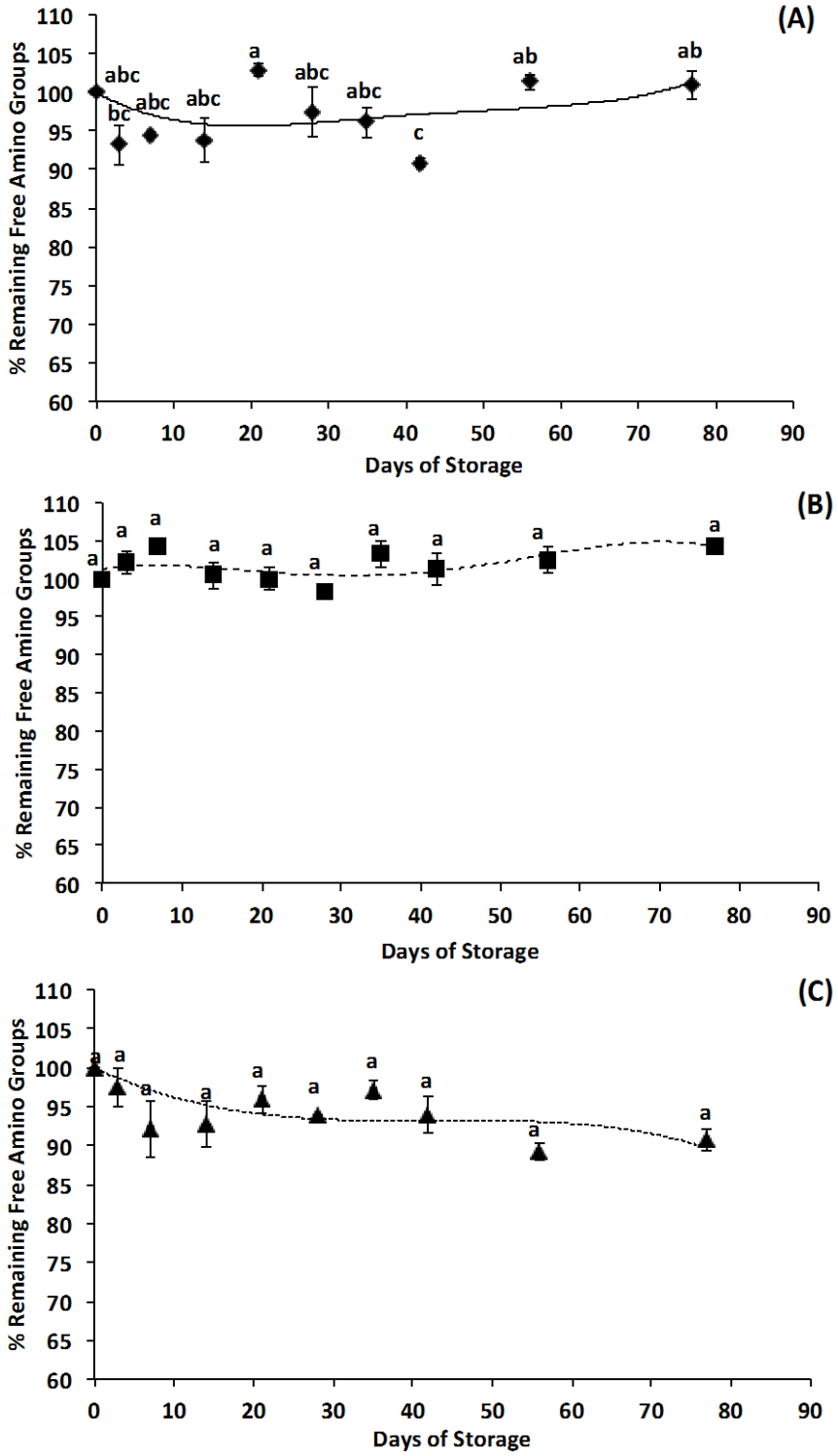

Figure 30. Change in % remaining free amino groups in SPI (A), SPH (B), and 50/50 (C) during storage at 0.05 a_w and 45°C. Error bars represent standard error (n=3). Lowercase letters above each point indicate significant difference between the means as determine following the Tukey-Kramer multiple means comparison test ($P \leq 0.05$).

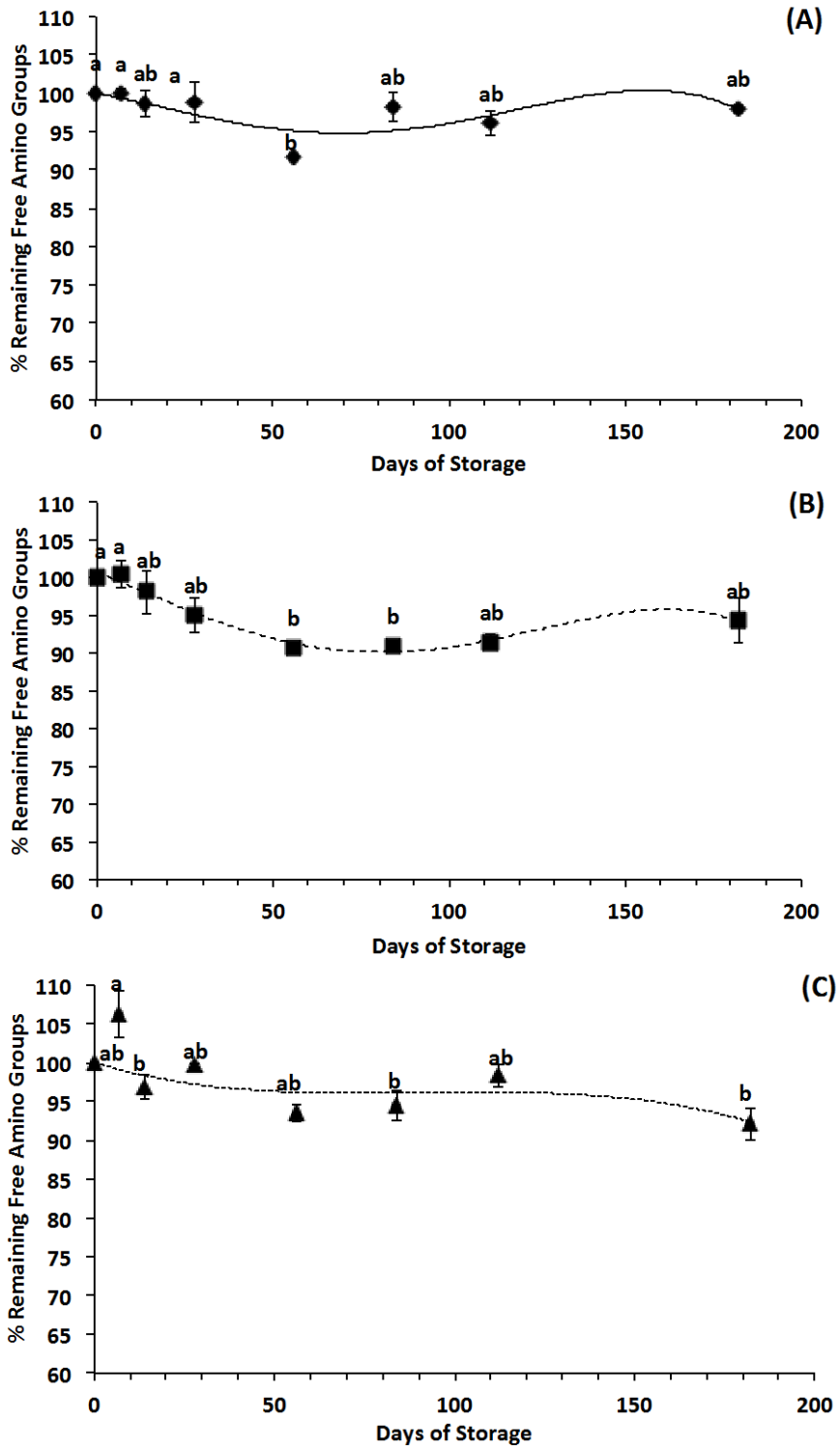

Figure 31. Change in % remaining free amino groups in SPI (A), SPH (B), and 50/50 (C) during storage at 0.74 a_w and 25°C. Error bars represent standard error (n=3). Lowercase letters above each point indicate significant difference between the means as determine following the Tukey-Kramer multiple means comparison test ($P \leq 0.05$).

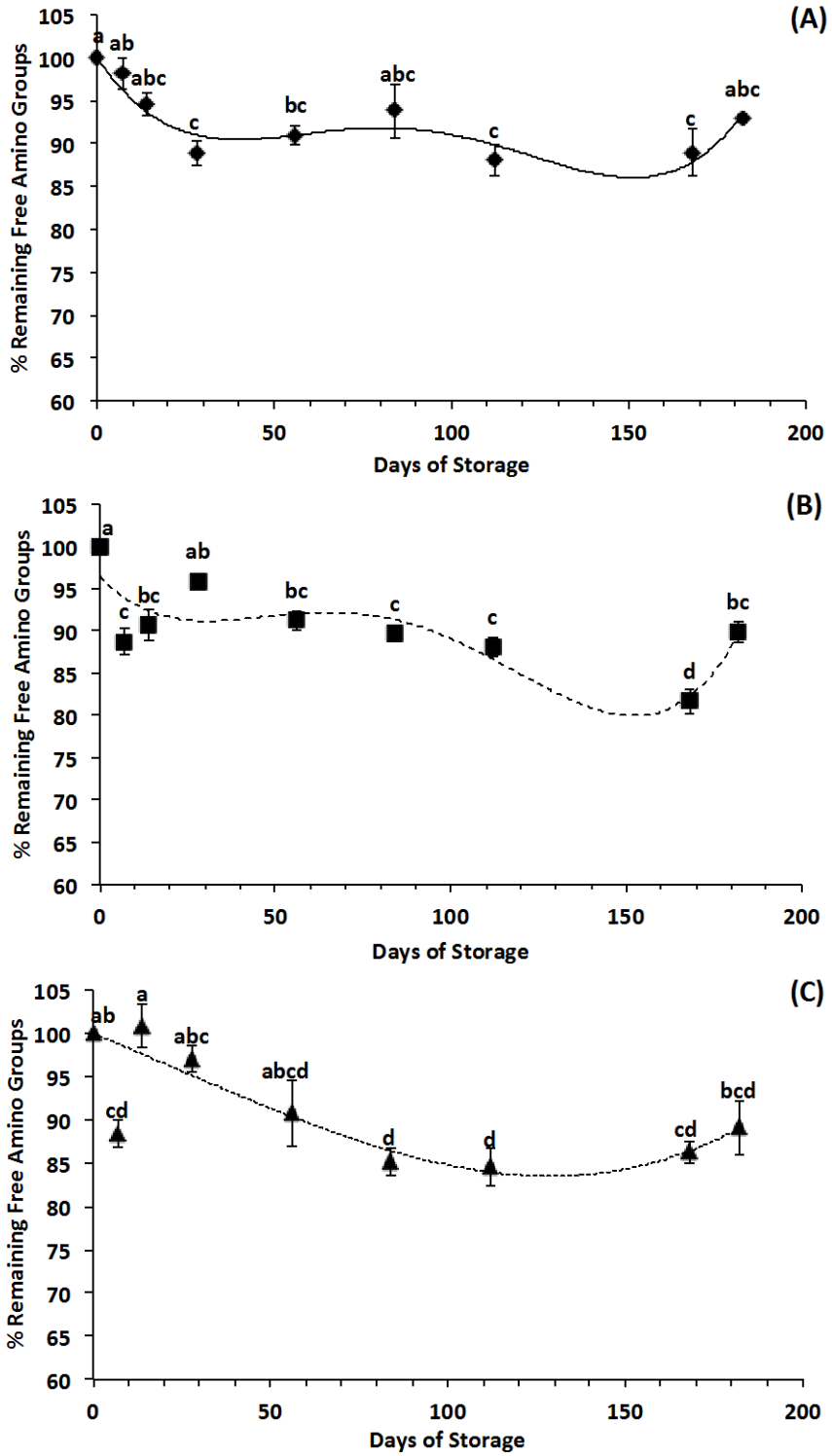

Figure 32. Change in % free amino groups in SPI (A), SPH (B), and 50/50 (C) during storage at 0.54 a_w and 25°C. Error bars represent standard error (n=3). Lowercase letters above each point indicate significant difference between the means as determine following the Tukey-Kramer multiple means comparison test ($P \leq 0.05$).

Appendix I: DSC Example Thermograms for Original SPI and Stored SPI

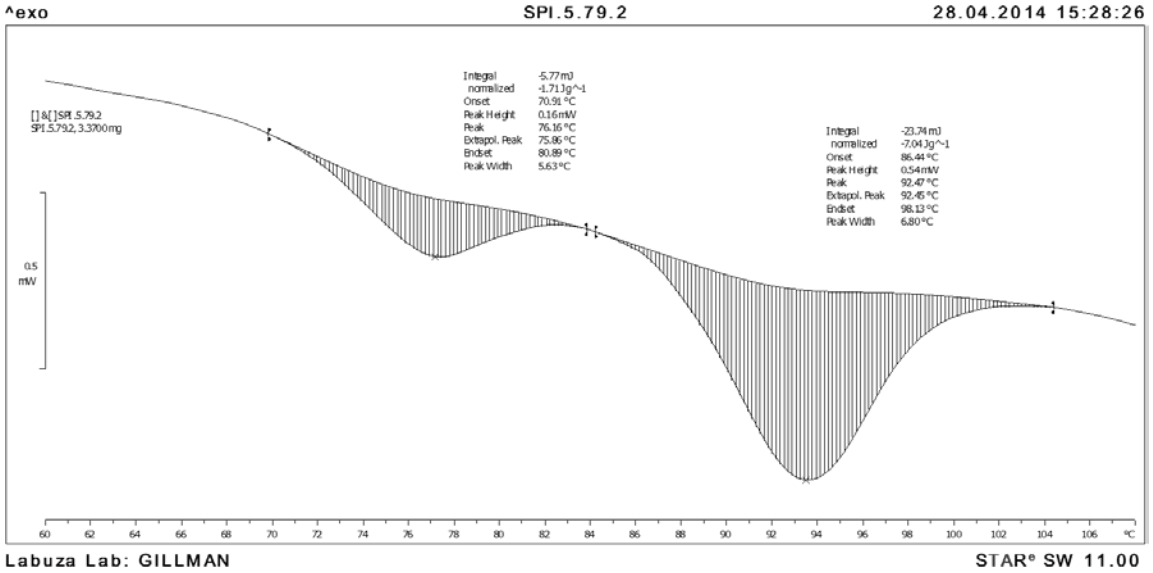

Figure 33. Thermogram of 20% dispersion of SPI (equilibrated to 0.79 a_w at 45°C, 5 days storage) in DDW. First endothermic peak corresponds to β-conglycinin, second peak corresponds to glycinin. Normalized integral is the change in enthalpy (ΔH) and peak is the denaturation temperature (T_d).

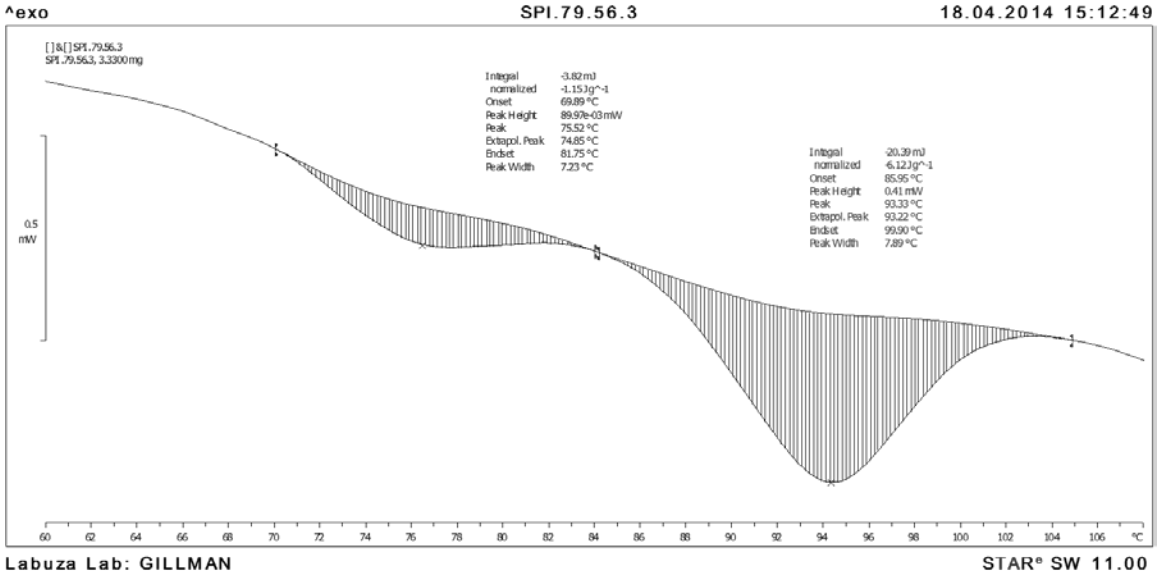

Figure 34. Thermogram of 20% dispersion of SPI (56 days at 0.79 a_w at 45°C) in DDW. First endothermic peak corresponds to β-conglycinin, second peak corresponds to glycinin. Normalized integral is the change in enthalpy (ΔH) and peak is the denaturation temperature (T_d).

Appendix J: Change in Fluorescence for SPI and 50/50 at 45°C

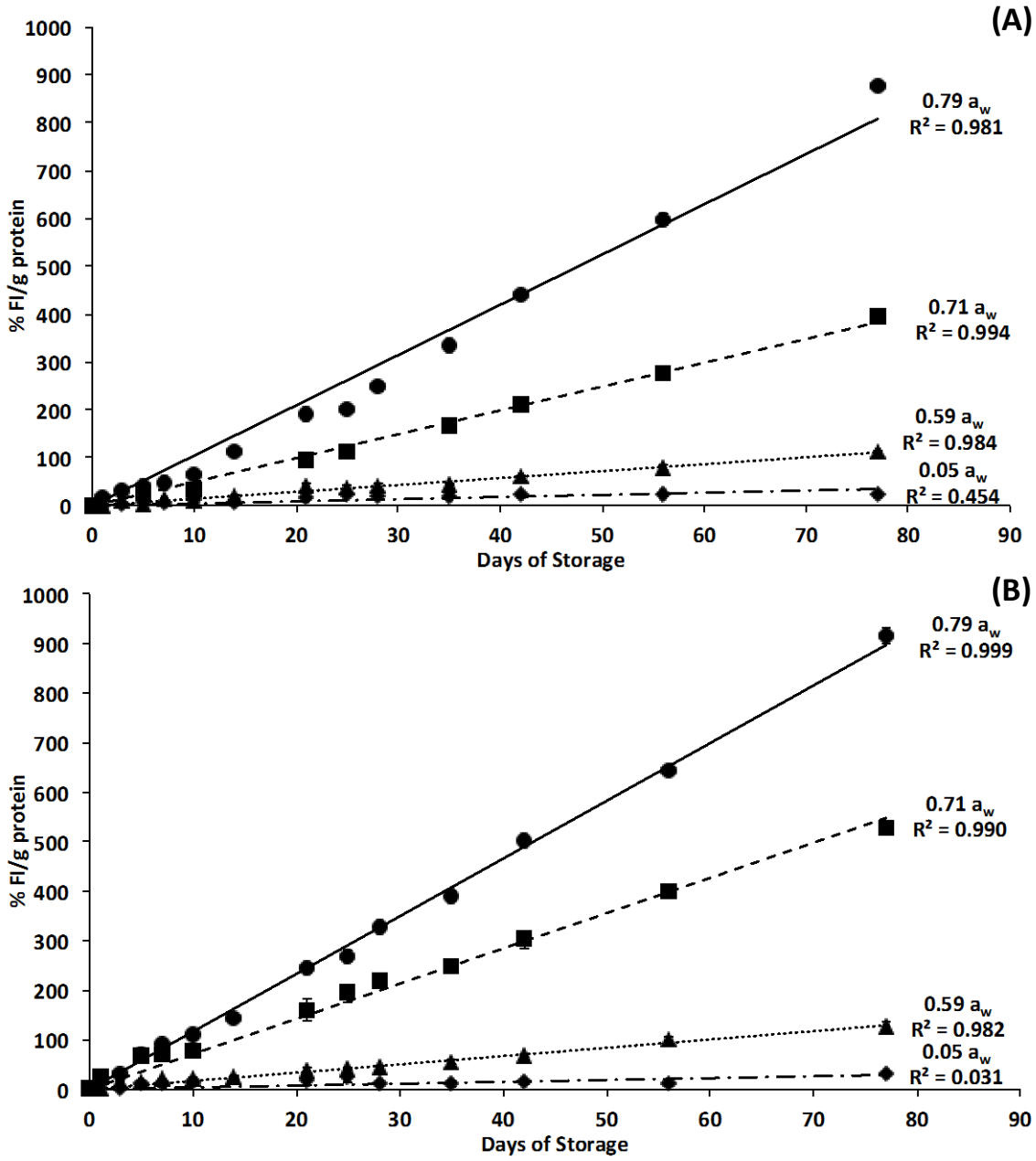

Figure 35. Change in fluorescence per gram protein over time at 45°C storage for SPI (A) and 50/50 (B) at 0.05, 0.59, 0.71, & 0.79 a_w. Error bars represent standard error (n=3). Goodness of fit (R^2) indicated for each regression.

Appendix K: Example Calculation for Percent Digestibility

Example calculation for the % digestibility of original (not stored) SPH.

Equation 12:

$$\%\ \text{Digestibility} = \frac{143031974 - 24932956}{143031974} * 100\%$$

$$\%\ \text{Digestibility} = 82.57\%$$

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

Table 11. Analysis of variance on the effect of sample type and a_w on overall color difference (ΔE) after 84 days at 45°C.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
SPI, SPH, & 50/50 at 0.05, 0.09, 0.23, 0.33, 0.43, 0.59, 0.71, & 0.79 a_w, 45°C	Sample type with a_w	23	29.370	239.704	0.000
	Error	45	0.123		

Table 12. Analysis of variance on the effect of a_w on solubility after 77 days at 45°C for SPI, SPH, and 50/50.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
SPI: original, 0.05, 0.09, 0.23, 0.33, 0.43, 0.59, 0.71, & 0.79 a_w, 45°C	a_w	8	3482.072	988.392	0.000
	Error	14	3.523		
SPH: original, 0.05, 0.09, 0.23, 0.33, 0.43, 0.59, 0.71, & 0.79 a_w, 45°C	Time	8	310.986	22.008	0.000
	Error	17	14.131		
50/50: original, 0.05, 0.09, 0.23, 0.33, 0.43, 0.59, 0.71, & 0.79 a_w, 45°C	Time	8	1112.058	95.614	0.000
	Error	16	11.631		

Table 13. Analysis of variance on the effect of a_w and time on solubility at 45°C for SPI, SPH, and 50/50.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
SPI: original, 0.05, 0.59, 0.71, & 0.79 a_w, 45°C	Time	12	3064.060	463.626	0.000
	Error	21	6.609		
SPH: original, 0.05, 0.59, 0.71, & 0.79 a_w, 45°C	Time	12	192.189	34.828	0.000
	Error	25	5.518		
50/50: original, 0.05, 0.59, 0.71, & 0.79 a_w, 45°C	Time	12	1207.085	142.188	0.000
	Error	22	8.489		

Table 14. Analysis of variance on the effect of time on the loss of free amino groups at 0.79 a_w at 45°C for SPI, SPH, and 50/50.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
SPI, 0.79 a_w, 45°C	Time	9	260.288	9.561	0.000
	Error	19	27.224		
SPH, 0.79 a_w, 45°C	Time	9	170.686	8.164	0.000
	Error	17	20.908		
50/50, 0.79 a_w, 45°C	Time	9	288.699	8.904	0.000
	Error	19	32.422		

Table 15. Analysis of variance of the effect of time on the loss of free amino groups at 0.71 a_w at 45°C for SPI, SPH, and 50/50.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
SPI, 0.71 a_w, 45°C	Time	8	30.215	0.866	0.561
	Error	18	34.878		
SPH, 0.71 a_w, 45°C	Time	9	106.224	5.112	0.002
	Error	18	20.780		
50/50, 0.71 a_w, 45°C	Time	9	98.808	7.843	0.000
	Error	20	12.598		

Table 16. Analysis of variance of the effect of time on the loss of free amino groups at 0.59 a_w at 45°C for SPI, SPH, and 50/50.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
SPI, 0.59 a_w, 45°C	Time	9	233.418	22.104	0.000
	Error	18	10.560		
SPH, 0.59 a_w, 45°C	Time	9	161.154	7.185	0.000
	Error	16	22.431		
50/50, 0.59 a_w, 45°C	Time	9	188.092	10.284	0.000
	Error	19	18.290		

Table 17. Analysis of variance on the effect of time on the loss of free amino groups at 0.05 a_w at 45°C for SPI, SPH, and 50/50.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
SPI, 0.05 a_w, 45°C	Time	9	49.098	4.766	0.002
	Error	20	10.301		
SPH, 0.05 a_w, 45°C	Time	9	11.560	1.951	0.102
	Error	20	5.926		
50/50, 0.05 a_w, 45°C	Time	9	30.498	2.300	0.063
	Error	18	13.260		

Table 18. Analysis of variance on the effect of time on the loss of free amino groups at 0.74 a_w at 25°C for SPI, SPH, and 50/50.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
SPI, 0.74 a_w, 25°C	Time	7	22.547	3.581	0.016
	Error	16	6.296		
SPH, 0.74 a_w, 25°C	Time	8	66.569	15.609	0.000
	Error	17	4.265		
50/50, 0.74 a_w, 25°C	Time	8	120.545	8.082	0.000
	Error	18	14.915		

Table 19. Analysis of variance on the effect of time on the loss of free amino groups at 0.54 a_w at 25°C for SPI, SPH, and 50/50.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
SPI, 0.54 a_w, 25°C	Time	8	53.286	5.447	0.001
	Error	18	9.782		
SPH, 0.54 a_w, 25°C	Time	7	47.841	4.747	0.005
	Error	16	10.079		
50/50, 0.54 a_w, 25°C	Time	7	61.075	6.963	0.001
	Error	16	8.771		

Table 20. Analysis of variance of the effect of a_w on the IC_{50} value of SPH and 50/50 after 77 days of storage at 45°C.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
SPH: original, 0.05, 0.59, 0.71, & 0.79 a_w	Sample type with a_w	7	0.028	15.684	0.000
	Error	16	0.002		

Table 21. Analysis of variance of the effect of a_w and sample type on the percent digestibility after 77 days of storage at 45°C.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
SPI: original, 0.05, 0.59, 0.71, & 0.79 a_w	a_w	4	248.051	7.349	0.009
	Error	8	33.753		
SPH: original, 0.05, 0.59, 0.71, & 0.79 a_w	a_w	4	125.922	3.794	0.045
	Error	9	33.127		
50/50: original, 0.05, 0.59, 0.71, & 0.79 a_w	a_w	4	223.213	3.647	0.050
	Error	9	61.201		