ABSTRACT

CRAMP, GRACE LOUISE. Modification and Molecular Interactions of a Soy Protein Isolate. (Under the direction of Dr. Christopher R. Daubert and Dr. Prachuab Kwanyuen).

Soy is a nutritional protein source with gelation and emulsification abilities that have expanded its use in the food industry. This research aimed to further the capabilities of soy protein by producing a readily reconstituted isolate capable of gelling at ambient temperatures. The modified soy protein isolate would have application in food systems requiring a high quality vegetarian protein and gelation ability without reaching required denaturation temperatures. Foods where the isolate may find use include a shelf stable soy protein based powder that may be used "on the go" to form a pudding type of dessert upon the addition of water.

Soy protein was modified with a heat treatment that denatured the protein at a concentration of 8% (w/w), required for gelation. The resulting soy protein isolate demonstrated immediate dispersibility and gelation in water, as well as heat stability during subsequent rheological thermal scans. The heat modified soy protein also remained two orders of magnitude higher in viscosity than an isolate that was originally denatured below a concentration required for gelation, after both of the isolates received a second thermal treatment at 8% protein (w/w). The viscosity difference between the two isolates demonstrates the importance of concentration at the point of denaturation, as irreversible denaturation may prevent interactions leading to preferable functionality at a later point.

The molecular interactions governing the gelation of the heat modified soy protein isolate were also investigated, as it is important to understand the network providing functionality for potential improvement. It was determined that while hydrophobic

interactions contribute to a majority of the network influencing final viscosity, intermolecular disulfide bonds must initiate network formation.

The heat modified soy protein isolate demonstrated an ability to conjugate to dextran (100-200 kDa) via Maillard reaction during a dry heat procedure. However, the conjugation of dextran (100-200 kDa) to the native soy protein isolate displayed a viscosity several orders of magnitude higher than the heat modified soy protein conjugate. The native soy protein isolate also showed a higher viscosity and greater ability as an emulsifier when conjugated to higher molecular weight polysaccharides such as dextran (100-200 kDa) than when conjugated to small molecular weight sugars such as mannose. Therefore, the heat modified soy protein isolate demonstrated gelation at ambient temperatures, whereas the high molecular weight soy protein conjugate demonstrated better emulsifying capabilities.

MODIFICATION AND MOLECULAR INTERACTIONS OF SOY PROTEIN ISOLATE

by

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DEDICATION

I would like to dedicate this thesis to Tricky Man Gone, my beautiful Quarter Horse, who remains a steady companion and showed me what it was like to fly without ever leaving the ground.

BIOGRAPHY

Grace Louise Cramp was born on October 20, 1981 in Reading, Pennsylvania. She is the daughter of James and Carolyn Cramp of Wernersville, Pennsylvania. She was the third generation in her family to attend Penn State University, and she graduated from Penn State with a Bachelor of Science in Food Science with highest distinction in 2004. After spending the summer of 2002 working in quality assurance for Turkey Hill Dairy in Lancaster, PA and the summer of 2003 working in product development for Hershey Foods in Lancaster, PA, Grace left the state where she grew up for NASA's Johnson Space Center in Houston, TX. After co-oping for NASA in the Space Food Systems Laboratory in the Fall 2003, Summer 2004, and Spring 2005, Grace moved to Raleigh, NC to pursue her Master of Science degree in Food Science at North Carolina State University under the direction of Dr. Christopher Daubert. After graduation, Grace will return to NASA for a fourth co-op rotation before pursuing a Ph.D. in Functional Genomics.

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PART I: Introduction

Soy is a high quality vegetarian protein source with associated nutritional benefits that have expanded soy's utility to the food industry in recent years. The U.S. soy crop value was greater than $17 billion in 2004, with $8 billion in soy and associated products exported. Around 40% of the crop was used in soy meal production, the majority of which is used in animal feed (Soy Stats, 2005). Soybean ingredients are currently used in many food products, including bakery foods, baby foods, confections, pastas, dairy, and snack foods (Soy Stats, 2005). Specifically, soy protein ingredients function in a range of applications in the food industry including foaming, fat absorption control, water absorption, gelation, and texture (Wolf, 1970). The health benefits of soy provide an opportunity to expand the functionality of soy ingredients and increase the use of soy products in food. Understanding the molecular implications incurred during soy protein processing can increase knowledge to improve or modify functional capabilities.

1.1 Soy Proteins

Soy protein ingredients currently include flours and grits (40-50% protein) (Horan, 1966; Tremple and Meador, 1958), concentrates (65-70% protein) (Meyer, 1966), and isolates (90% protein and greater) (Meyer, 1966). In a commercial setting, proteins may become completely denatured during isolation (Hermansson, 1978; Wagner et al., 1992). Commercially prepared soy protein isolates have been shown to provide a range in solubility, functionality and viscosity depending on degree of denaturation, method of processing, or other unknown causes (Nash and Wolf, 1967; Hermansson, 1978; Hermansson, 1986; Wagner et al., 1992; Chronakis, 1996). Wagner et al. (1992) suggested that the denatured soy protein formed insoluble aggregates that would not interact in new structures. The differences in functionality demonstrate the importance of understanding the modification

process in relation to structure formation of a particular isolate to tailor soy protein ingredients to a specific application.

As the use of soy protein ingredients has increased, the number of patents commercializing on the functional aspects of soy protein gelation are on the rise in an attempt to capitalize on the functionality produced by processing differences. A thermally processed high molecular weight soy protein isolate has been described to have high water solubility and low sedimentation and viscosity, with applications in emulsification for beverages and meat analogs (Monagle et al., 2002). In this patent, isolate production may incorporate a concentrating step with membrane filtration, however, the filtration step comes after an initial thermal process. The temperatures used (110-121°C) have been shown to lead to gel softening, higher protein solubility, and loss of disulfide bonds (Shimada and Cheftel, 1988). The actual molecular network forming the large molecular weight aggregates in the patent was not explained, leaving an opportunity to understand the formation of the aggregates and the increase in protein solubility (Monagle et al., 2004).

Traditionally soy protein is a thermally-gelling protein. However, cold set gelation of soy protein gels at ambient temperature has been demonstrated using a preheating step to denature the proteins, followed by addition of calcium to induce gelation through electrostatic interaction (Maltais et al., 2005). This example of cold gelation demonstrates the potential for modifications to soy protein isolate (SPI) to improve and expand on current functional capabilities. An understanding of soy protein and current thermal-gelation mechanisms is required to increase SPI functionality through modification.

Soy protein functionality may also be increased by conjugation with polysaccharides via the Maillard reaction (Cabodevila et al., 1994; Diftis and Kiosseoglou, 2004; Diftis et al.,

2005). However, there is opportunity to increase the range of functional performances through carbohydrate selection prior to conjugation.

1.1.1 Soy Protein Fractions and Subunits

There are four major fractions of soy proteins, based on ultracentrifugal sedimentation rates, designated by Svedberg unit: 2S, 7S, 11S, and 15S. The 2S fraction accounts for 22% of the water extractable soy protein and contains trypsin inhibitors and cytochrome c (Wolf et al., 1962; Steiner and Frattali, 1969; Fridman et al., 1968). The 7S fraction accounts for 37% of the water extractable soy protein, the majority of which is β-conglycinin (7S) with a molecular weight of 140-210 kDa, containing 3 major subunits, α', α, and β with molecular weights of 58 kDa for α', 57 kDa for α, and 42 kD for β (Wolf et al., 1962; Thanh and Shibasaki, 1977; Thanh and Shibasaki, 1978; Koshiyama, 1968). Glycinin (11S) makes up 31% of the protein, and consists of the major soybean reserve protein, 11S globulin (glycinin), with a molecular weight around 320-375 kDa (Wolf and Briggs, 1959; Wolf et al., 1962; Utsumi et al., 1981; Badley et al., 1975). The 11S fraction consists of six subunits of disulfide bonded acidic and basic polypeptide chains. When the disulfide bonds are broken, the individual acidic polypeptides are around 35-38 kDa and the basic polypeptides are 18-20 kDa (Utsumi et al., 1981; Badley et al., 1975). The 15S fraction makes up 11% of the protein. Overall, 80% of the proteins have a molecular weight greater than 100,000 (Wolf, 1970). The soy proteins are insoluble at their isoelectric point (pH 4.3-4.5), aiding in protein extraction processes (Wolf, 1970; Lusas and Rhee, 1995).

1.1.2 Soy Protein Gelation and Associated Molecular Interactions

The function of the 7S and 11S components and their subunits in soy protein gelation has been investigated as separate components and in combination. The subunits that

aggregate and become insoluble during thermal processing contribute mostly to gelation functionality (Wolf, 1970; Petrucelli and Anon, 1995a.; Petrucelli and Anon, 1995b.; Sorgentini et al., 1995). At greater than 8% soy protein isolate powder the α', α, and β subunits became insoluble during heating (Sorgentini et al., 1995). The basic subunit was also observed to aggregate and become insoluble during heating (Wolf, 1970; German et al., 1982). The basic subunit and the β subunit were reported to become insoluble during heating at 0.5% protein concentration (Utsumi et al., 1984), likely due to aggregation between the subunits after the 11S disulfide bonds are reduced and the basic subunit is free to interact (Petrucelli and Anon, 1995b). The concentration dependence (8% protein) found for some subunits to aggregate and lose solubility has also been observed to be the "critical concentration" for gelation. Soy protein isolate was observed to form a gel during heating above a concentration of 7-8% soy protein (Circle et al., 1964; Sorgentini et al., 1995; Catsimpoolas and Meyer, 1970). Circle et al. (1964) showed exponential increases in apparent viscosity with concentration for both unheated and heated gels. Gelation occurred at concentrations of 7% soy protein and greater during heat denaturation, which is the point where a continuous network forms.

The protein chemistry involved in gelation may involve many interactions, including electrostatic interactions and hydrogen bonds, hydrophobic interactions, covalent bonds, and ionic bonds. Soy protein structure changes during denaturation provide the opportunity for gelation interactions to occur.

1.1.2.1 Electrostatic Interactions and Hydrogen Bonds

Electrostatic interactions are collectively known as van der Waals forces and include dipole-dipole forces, London dispersion forces, and hydrogen-bonding. These forces are less than 1/6 as strong as covalent or ionic bonds (Brown et al., 2000).

London dispersion forces are momentary, weak interactions that can occur in proteins between two molecules when one instantaneous dipole moment induces an adjacent instantaneous dipole moment in an adjacent molecule (Brown et al., 2000) (figure 1.1).

Figure 1.1. London dispersion forces – induced dipoles.

Hydrogen bonds are stronger interactions in proteins, between the hydrogen atom in a polar bond and the unshared electron pair in a nearby electronegative ion or atom (Brown et al., 2000). In proteins, hydrogen bonds can exist between the amino groups of proteins and water (figure 1.2), or between proteins.

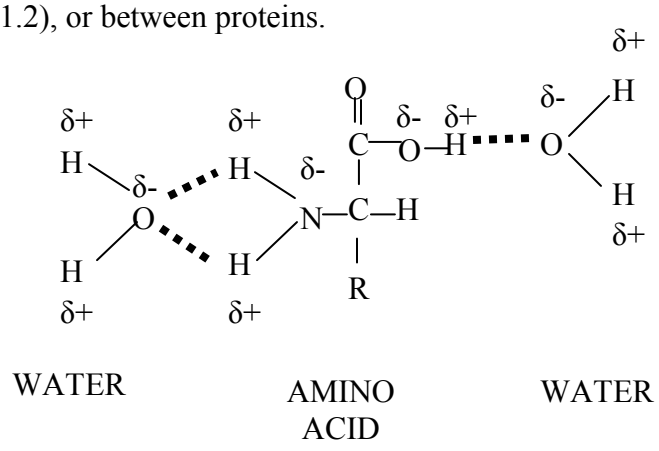

Figure 1.2. Hydrogen bonding between the amine group on an amino acid and water and a carboxylic group on an amino acid and water.

1.1.2.2 Hydrophobic Interactions

Hydrophobic interactions are even stronger than hydrogen bonds, and are due to the repulsion of water by hydrophobic molecules. The hydrophobic amino acid side chains associate with each other rather than with water, decreasing the number of water molecules required to form an ordered structure around the hydrophobic groups, increasing the entropy of the system (Brown et al., 2000) (figure 1.3).

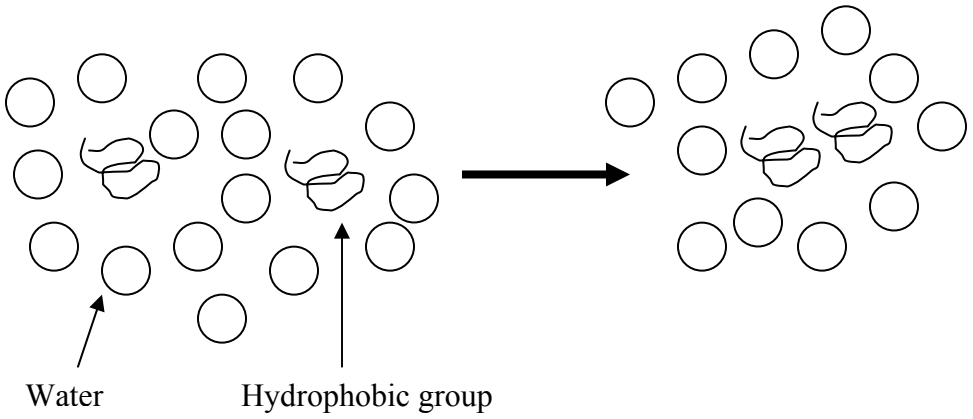

Figure 1.3. Hydrophobic interaction.

1.1.2.3 Covalent Bonds

Covalent bonds are very strong and involve a chemical link between molecules (Brown et al., 2000). Disulfide bonds are covalent bonds that may break and form under appropriate thermal conditions, such as the disulfide bonds between 11S acidic and basic subunits in soy protein (figure 1.4) (Wolf, 1993).

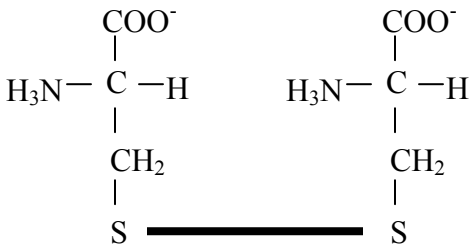

Figure 1.4. Covalent disulfide bond between two cysteine residues.

1.1.2.4 Ionic Bonds

Ionic interactions may occur in proteins when salts are present. These are very strong and are created by the attraction between the positive and negative charges on salts (Na^+, Cl^-, K^+, Ca^{2+}, and so on) (figure 1.5) (Brown et al., 2000). The charges created by salts can affect the strength of many gels. For example, calcium salts interact with soy protein to form tofu.

$$Na^+ \bullet\bullet\bullet\bullet Cl^-$$

Figure 1.5. Ionic bond between sodium and chloride in salt.

1.1.2.5 Effect of Denaturation on Interactions

When proteins denature and aggregate due to heating, unless covalent linkages occur the structures should eventually renature upon cooling. Renaturation can be prevented through intermolecular cross-linking between side groups. Sulfur bridges could also rearrange through sulfhydryl/disulfide interchange after breaking at high temperatures,

creating intermolecular bonds in the aggregated protein molecules where intramolecular bonds once existed. Sulhydryl/disulfide interchange would not necessarily change the total number of disulfide bonds (Walstra, 2003).

The molecular interactions involved in gelation may vary based on the soy protein subunits involved, which may depend on the temperature involved in gelation, and the resulting degree of denaturation. The temperature of denaturation ranges from 68-82°C for 7S and 83-95°C for 11S (Sorgentini, et. al. 1995; German et al., 1982; McKlem, 2002). The denatured subunits may aggregate and become insoluble (Wagner et al., 1992), resulting in a protein gel with different interactions depending on the subunits involved. The molecular interactions of proteins can be elucidated by the use of several reducing or denaturing agents.

1.1.3 Elucidating Molecular Interactions in Soy Protein Gelation

Denaturants that interfere or interact with different types of bonds may be used to provide insight into the molecular interactions involved in a network. Changes in viscosity or firmness upon the addition of a denaturant may indicate the presence of a type of bond in the network, as well as its importance.

1.1.3.1 Electrostatic Interactions, Including Hydrogen Bonds

Electrostatic interactions may be determined by measuring the effects of denaturants that neutralize charge, such as sodium chloride (NaCl) and sodium thiocyanate (NaSCN). The salts NaCl and NaSCN decreased the firmness of 11S gels (Utsumi and Kinsella, 1985) and 7S gels (Nagano et al., 1994), and increased the solubility of 7S, 11S, and soy protein isolate (Utsumi and Kinsella, 1985), revealing the contribution of electrostatic interactions, including hydrogen bonds.

1.1.3.2 Hydrophobic Interactions and Hydrogen Bonds

Protein hydrophobic interactions and hydrogen bonds can be destabilized by urea and guanidinium hydrochloride. Urea is commonly used to denature proteins, but the mechanism is not clearly understood. Wallqvist et al. (1998) found that urea denatures a protein molecule through preferential adsorption with charged protein solutes, dehydrating the molecules and causing repulsion between proteins, stabilizing the unfolded form. Zou et al. (1998) reported that urea binds to amide groups through hydrogen bonds, decreasing the hydrophobic effect through dehydration of the protein molecule, and indicating hydrophobic groups and hydrophilic groups are involved with urea denaturation. Walstra (2003) also reported that the denaturing effect is caused by a dehydration of peptide bonds as urea binds to them, weakening hydrophobic interactions. Therefore, urea likely interferes with both hydrogen bonding and hydrophobic interactions by dehydrating a molecule and interacting through hydrogen bonds that might otherwise interact with the solvent surrounding the molecule.

Low concentrations of detergent or urea resulted in soy protein conformational changes (Wolf and Briggs, 1958). The 7S subunits α, α', and β were found to associate mainly through hydrophobic interactions (Thanh and Shibasaki, 1978). The resulting trimeric glycoprotein contains mannose and glucosamine (Thanh and Shibasaki, 1978; Koshiyama, 1969). Nagano et al. (1994) also found that hydrophobic interactions were important in 7S gelation, but the findings also included a significant contribution from hydrogen bonds. The presence of hydrogen bonds was supported through thermoreversibility of soy protein gels (Nagano, 1994; McKlem, 2002). Utsumi and Kinsella (1985) also demonstrated the importance of hydrogen bonds in SPI gels. German et al. (1982) found a

significant contribution from hydrophobic interactions in 11S gelation. Hydrophobic interactions have been shown to become important at lower gelation temperatures (80°C), where only the cysteine deficient 7S fraction was denatured (Catsimpoolas and Meyer, 1970).

1.1.3.3 Sulfhydryl/disulfide Interchange

Sulfhydryl/disulfide interchange during protein denaturation and aggregation was described in 1959 (Jensen, 1959). This method proposed that free sulfhydryl groups could interchange with a disulfide linked group, forming a new disulfide bond and a new sulfhydryl group, a reaction often occurring during protein denaturation. The interchange may result in intermolecular covalent bond formation where an intramolecular covalent bond had existed, creating a protein network (figure 1.6) (Walstra, 2003).

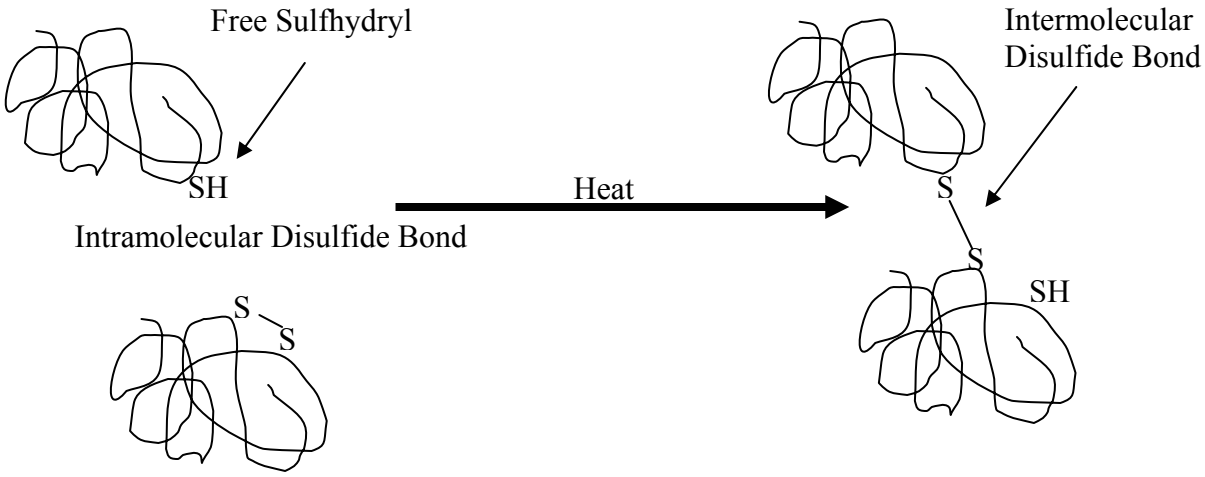

Figure 1.6. Schematic of sulfhydryl/disulfide interchange from intramolecular disulfide bond to intermolecular disulfide bond.

Sulfhydryl/disulfide interchange has been proposed as a mechanism for soy protein gelation based on the reaction of the gel to several reagents: cysteine (Circle et al., 1964;

Wang and Damodaran, 1990), β-mercaptoethanol (β-ME) (Briggs and Wolf, 1957; Wolf and Briggs, 1958; Catsimpoolas and Meyer, 1970; Utsumi et al., 1984; Utsumi and Kinsella, 1985; Wolf, 1993), dithiothreitol (DTT) (Utsumi and Kinsella, 1985; Wolf, 1993; McKlem, 2002), N-ethylmaleimide (NEM) (Briggs and Wolf, 1957; Wolf and Briggs, 1958; Catsimpoolas and Meyer, 1970; Utsumi and Kinsella, 1985; Shimada and Cheftel, 1988; Wang and Damodaran, 1990), iodoacetamide (Briggs and Wolf, 1957; Wolf, 1993), sodium borohydride ($NaBH_4$) (Wolf, 1993), and sodium sulfite (Na_2SO_3) (Circle et al., 1964; Petrucelli and Anon, 1995b). Previous investigations of disulfide bonds and sulfhydryl groups in soy protein have indicated most opportunities for disulfide bonding are in the 11S fraction (Shimada and Cheftel, 1988; Thanh and Shibasaki, 1977; Koshiyama, 1971; Utsumi and Kinsella, 1985). Sulfhydryl groups provide the potential for irreversible covalent interactions. The 7S fraction contains four sulfhydryl groups that participate in two intramolecular disulfide bonds, whereas 11S has 48 sulfhydryl groups, many of which interact in bonds between the acidic and basic subunits (Koshiyama, 1971; Hermansson, 1978).

1.1.4 Contribution of Each Molecular Interaction in Soy Protein Gelation

Although most researchers agree that sulfhydryl/disulfide interchange plays a part in gelation due to the effect of disulfide reducing agents, their actual role with respect to other molecular interactions has not been fully established and may depend on gelation temperature and protein concentration. Wolf and Briggs (1958) reported that 11S could dissociate with β-ME and NEM, resulting in a change in conformation. Circle et al. (1964) observed that soy protein gels are irreversible under heating conditions of 100°C for 30 minutes, but disulfide reducing agents prevented gelation, forming the conclusion that

sulfhydryl/disulfide exchange must be a part of the gelation mechanism. Alternatively, Catsimpoolas and Meyer (1970) proposed a schematic involving a sol, progel, and gel, where the gel, formed at 80°C, was reversible and therefore must be formed through noncovalent interactions. In addition, Catsimpoolas and Meyer (1970) found that small amounts of reducing agents reduced gel strength, but large amounts allowed the gel to unfold and create more preferential interactions. However, further support for disulfide bonds was demonstrated when SPI gels were shown to increase in firmness with temperature up to 120°C, coincident with a decrease in free sulfhydryl groups and protein solubility (Shimada and Cheftel, 1988).

Utsumi and Kinsella (1985) found that sulfhydryl/disulfide exchange was involved in the elasticity of SPI gels, but not necessary for gel formation. Alternatively, Wang and Damodaran (1990) reported that the increase in molecular weight due to intermolecular disulfide bond formation resulted in gelation by creating a network capable of entanglement. Further debate as to the role of disulfides was provided when McKlem (2002) revealed that disulfides are important in the native protein, but become less significant post thermal treatment, once hydrophobic interactions occur.

Disulfide-linked polymers of the 11S fraction were also found to form due to air oxidation during isolation (Briggs and Wolf, 1957). These fractions were shown to depolymerize with the addition of mercaptoethanol, simultaneously increasing solubility. Polymerization was found to occur when the protein was in the precipitated, reduced state, and was prevented by NEM and iodoacetamide.

Sulfhydryl-disulfide interchange from intramolecular to intermolecular disulfide bonds has been stated as the interaction forming water-insoluble edible barrier coatings and

films in heated soy protein isolates (Krochta and McHugh, 1996). The temperatures required to form these patented coatings and films ranged from 70-95°C and heat treatments times ranged from 15 minutes to 3 hours.

Native thaumatin proteins are commercially available natural sweeteners that do not contain a free sulfhydryl group. However, disulfide rearrangement has occurred in thaumatin proteins when heated above 70°C for 15 minutes, resulting in aggregation and loss of sweetness and demonstrating the ability of disulfides to break under appropriate thermal conditions. Iodoacetamide and NEM suppressed the disulfide rearrangement and prevented insoluble thaumatin aggregates from forming via intermolecular disulfide bonds (Kaneko and Kitabatake, 1999). The disulfide exchange in the absence of a free sulfhydryl group demonstrates an increased potential for rearrangement at high temperatures, where disulfides break.

1.2 Functionality Through Carbohydrate Conjugation via Maillard Reaction

Soy protein functionality may also be increased by conjugation to polysaccharides via the Maillard reaction (Cabodevila et al., 1994; Diftis and Kiosseoglou, 2004; Diftis et al., 2005). There is opportunity to increase the range of functionalities through carbohydrate selection prior to conjugation. An understanding of the Maillard reaction would facilitate choice in carbohydrate and conjugation conditions.

1.2.1 Maillard Reaction

The Maillard reaction is a nonenzymatic browning reaction between a reducing sugar and an amino acid (figure 1.7). First observed by Louis Maillard in 1912, the reaction results in the formation of dark brown colors, flavors, and aromas (Izydorczyk, 2005).

Nonenzymatic browning reactions are desirable in foods such as cookies and cakes. However, in other food systems there is a concern with the formation of carcinogens (Izydorczyk, 2005) or the loss of the essential amino acid, lysine (Nursten, 2005), as the proteins and amino acids react with sugars.

The Maillard reaction begins with a carbonylamine reaction when the amino group of an amino acid reversibly condenses with the carbonyl group of a reducing sugar (figure 1.7.a). Subsequently, water is formed when the molecule forms a Schiff's base (figure 1.7.b). An N-substituted glycosylamine is formed when the Schiff's base cyclizes (figure 1.7.c) (Nursten, 2005; Izydorczyk, 2005). Digestible lysine is not lost in the beginning stages of the Maillard reaction, as it is still bioavailable when bound in glycosylamine (Nursten, 2005).

The cyclization is rapidly followed by an irreversible, acid catalyzed, Amadori rearrangement to convert the unstable aldose form to a ketose form, the N-substituted 1-amino-2-deoxy-2-ketose Amadori compound (figure 1.7.d) (Izydorczyk, 2005; Nursten, 2005). The Amadori rearrangement may take place spontaneously, as its products have been found at room temperature, and even in frozen products. Amadori compounds bind lysine so it is no longer bioavailable (Nursten, 2005).

Many complex flavor compounds may then be formed depending on the reaction route the Amadori compound takes. Flavor compounds include melanoidins, nitrogenous polymers that provide the brown color characteristic of the Maillard reaction. At a pH below 7.0, amino-deoxy-ketose may proceed through 1,2-enolization to form 1,2-enaminol, which eventually results in furfural and hydroxymethylfurfural formation (Izydorczyk, 2005; Nursten, 2005). These products condense with amino acids and polymerize to form the

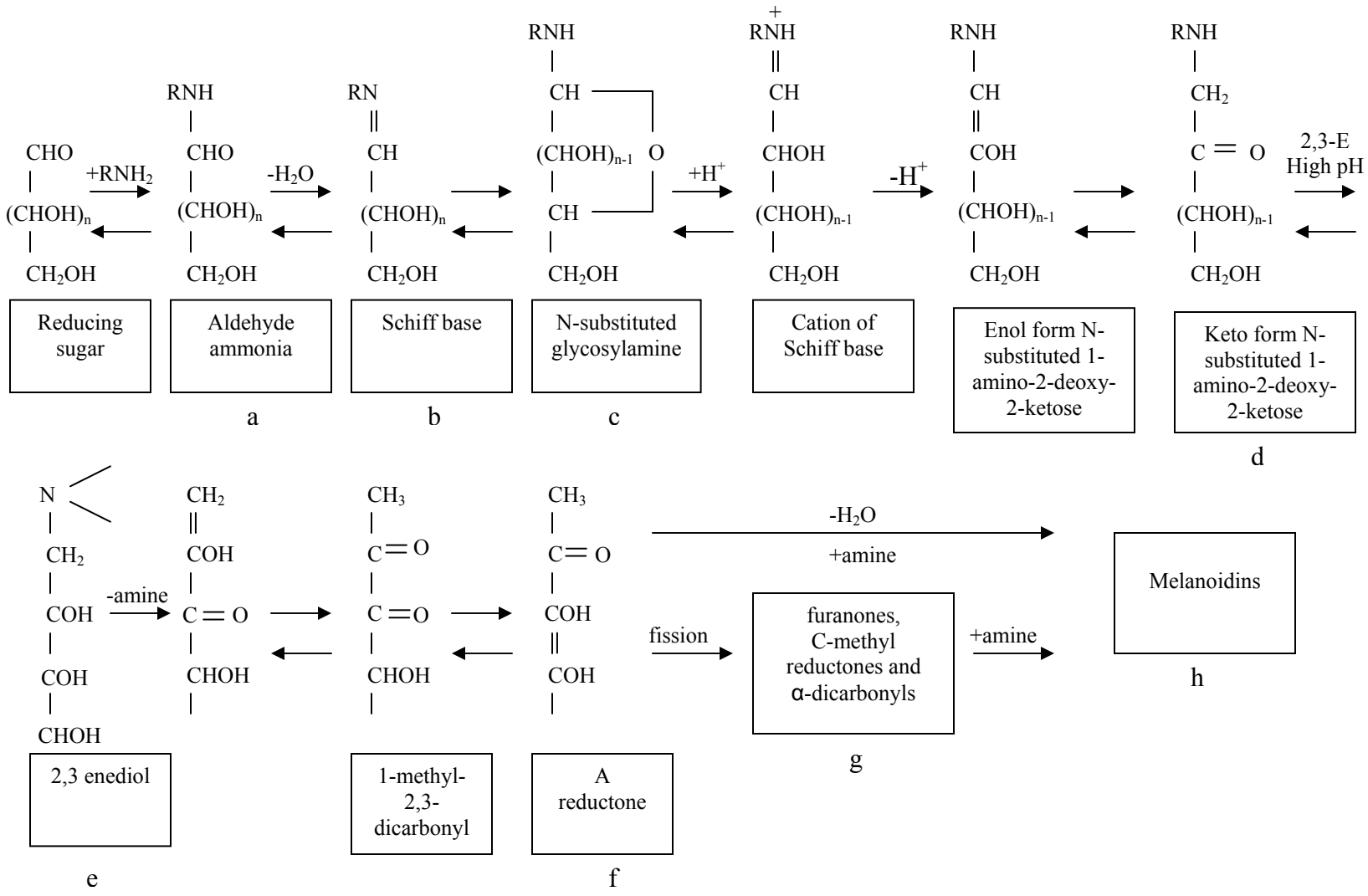

Figure 1.7. Maillard reaction for protein and reducing sugar combinations around pH 7, modified from Nursten (2005).

melanoidins (Izydorczyk, 2005). At a lower pH, the Amadori compound takes longer to decompose and less browning is evident than at a higher, more neutral pH (Nursten, 2005).

At a high pH, 2,3-enolization takes place at a different position on the amino-deoxy-ketose molecule and 2,3-enediols are formed (figure 1.7.e). Enediols lead to the production of reductones (figure 1.7.f). This reaction produces dicarbonyl intermediates that form flavor compounds (aldehydes, furans, maltol, isomaltol, and diacetyls) when they decompose, dehydrate, or fragment (figure 1.7.g). Melanoidins may also be produced when reductones interact with amino acids (figure 1.7.h) (Izydorczyk, 2005).

Finally, flavor compounds may be produced through Strecker degradation, beginning when dicarbonyl intermediates formed from the production of reductones are present during amino acid oxidization and form aldehydes. Flavor compounds result when the aldehydes form melanoidins or are involved in condensation reactions to form heterocyclic compounds, pyrazines, pyrrolines, oxazole, oxazolines, and thiazole derivatives (Izydorczyk, 2005).

1.2.1.1. Maillard Reaction Conditions

The Maillard reaction is regulated by water activity, type of amino acids available, and type of sugar present. Different sugars exhibit different rates of reactions and reactivity depending on how fast the ring opens. For example, pentoses are more reactive than hexoses. When considering hexoses, galactose is more reactive than mannose, which is more reactive than glucose (Izydorczyk, 2005).

Generally, nonenzymatic browning occurs optimally in food at water activities of 0.3 to 0.7. Decreasing water activity generally increases the browning rate in most food systems, except in foods where it limits the mobility of the amino acids and reducing sugars (Eichner and Karel, 1972). The reaction may proceed more rapidly at lower water activities because

water is a byproduct of several intermediate reactions (Izydorczyk, 2005) and high water activities may also dilute the reactants (Stamp and Labuza, 1983).

1.2.2 Reducing Sugars

Different sugars are expected to have different functionalities when conjugated to protein. Polysaccharides may have fewer reducing ends per molecular weight, and so have fewer chances to conjugate, but the longer chain may increase steric effects and improve emulsifying capabilities of proteins (Dickinson and Galazka, 1991). Smaller sugars with more reducing ends per molecular weight may have the potential to conjugate to a greater extent, but lack the large hydrophilic chains extending from the molecule. Dextran and mannose are a long chain polysaccharide and a simple sugar, respectively.

1.2.2.1 Dextran

Dextran is a branched polysaccharide that ranges in molecular weight distribution and may be produced from sucrose by the microorganisms *Leuconostoc mesenteroides streptococcus* and *Streptococcus mutans* (Wikipedia, 2007). A segment of a dextran structure is shown in figure 1.8. Dextran may also contain α-(1→2) and α-(1→4) glucose linkages. The (1→6) linkage increases water solubility (Izydorczyk 2005). Each dextran molecule has one reducing end for reaction with amine groups (Dickinson and Semenova, 1992).

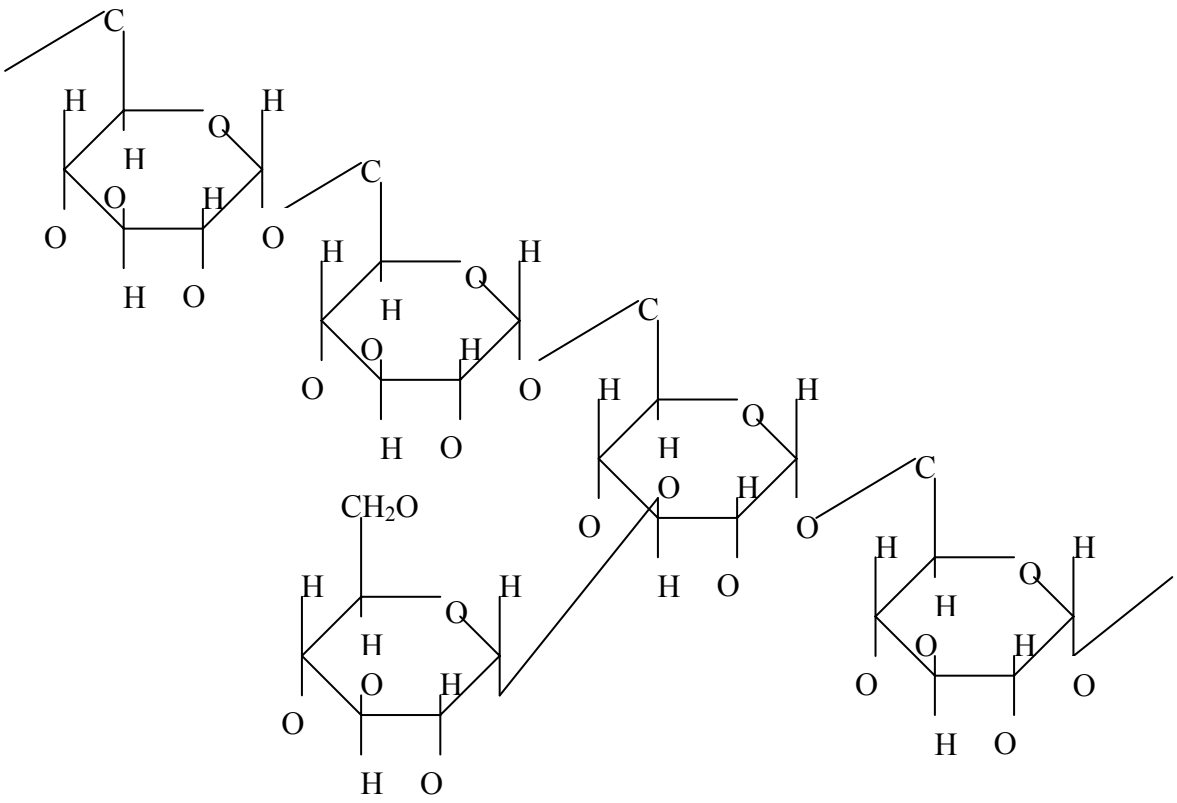

Figure 1.8. Structure of dextran.
(modified from Pharmacosmos A/S, 2007)

1.2.2.2 Mannose

Mannose is a simple six carbon monosaccharide with a reducing end that may participate in the Maillard reaction (figure 1.9). Each hexose has a reducing end, providing the potential for more reactivity via the Maillard reaction than larger polysaccharides with fewer reducing ends or steric hindrances (Izydorczyk, 2005). However, the high molecular weight and branching of dextran may provide the opportunity to increase the functionality of proteins when conjugated through the Maillard reaction (Kato et al., 1988).

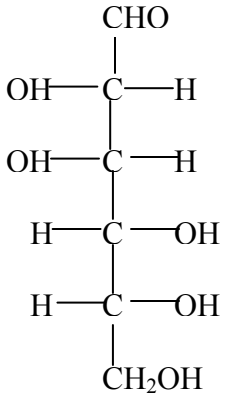

Figure 1.9. Structure of D-Mannose.

1.2.3 Emulsions and Soy Proteins

The ability of a soy protein conjugate to act as an emulsifier may be defined in terms of emulsifying capacity, emulsifying stability, or emulsifying activity of the final emulsion (Pearce and Kinsella, 1978). Soy protein must have surface active features to function as an emulsifier and lower the interfacial tension of dispersed oil droplets in a continuous phase (Walstra, 2003). Soy proteins contain amphiphilic features allowing them to interact with both a hydrophobic phase and a hydrophilic phase (Voutsinas et al., 1983).

1.2.3.1 Emulsifying Capacity (EC)

Emulsion capacity is defined as the maximum amount of oil that a standard amount of protein can emulsify under specified conditions (Pearce and Kinsella, 1978). Emulsion capacity is a function of the equipment, method, and other conditions (pH, type of oil, temperature), not just the type or quantity of protein. Therefore, EC is a qualitative test useful when comparing the emulsifiers under the same conditions (Tornberg and Hermansson, 1977; Carpenter and Saffle, 1964). Swift et al. (1961) developed a method to measure emulsifying capacity by adding oil at an established rate into a blender with the

protein solution, producing an oil-in-water emulsion, and visually determining when the emulsion collapsed. The EC was reported as the amount of oil emulsified by 100 grams of protein. Subsequently, Webb et al. (1970) modified the method by Swift et al. (1961) and used electrical conductivity to objectively determine the emulsifying capacity of several muscle proteins. A sudden spike in the blender amperage represented the point where the emulsion destabilized. The method is based on the ability of water and the inability of oil to conduct electricity (Webb et al., 1970). Firebaugh (2004) compared the EC of a commercial whey protein concentrate and a modified whey protein isolate using the electrical conductivity method, and determined there was no difference in EC between the two systems. Firebaugh (2004) used the setup exhibited in figure 1.10 to measure EC.

1.2.3.2 Emulsifying Stability (ES)

Emulsion stability is a measure of the oil separation from an emulsion over a standard period of time (Pearce and Kinslla, 1978). Emulsion stability may be measured with a creaming index by relating the height of the emulsified layer to the height of the original emulsion (Equation 1) (Keowmaneechai and McClements, 2002).

$$\text{creaming index} = 100 \times (H_s/H_e) \qquad \text{Equation 1}$$

1.2.3.3 Emulsifying Activity

A method to determine emulsifying activity in relation to the interfacial area of an emulsion has previously been developed by Pearce and Kinsella (1978). The method employed the measurement of emulsion turbidity with absorbance. An increase in the measurement of turbidity, through absorbance, was attributed to an increase in emulsifying activity.

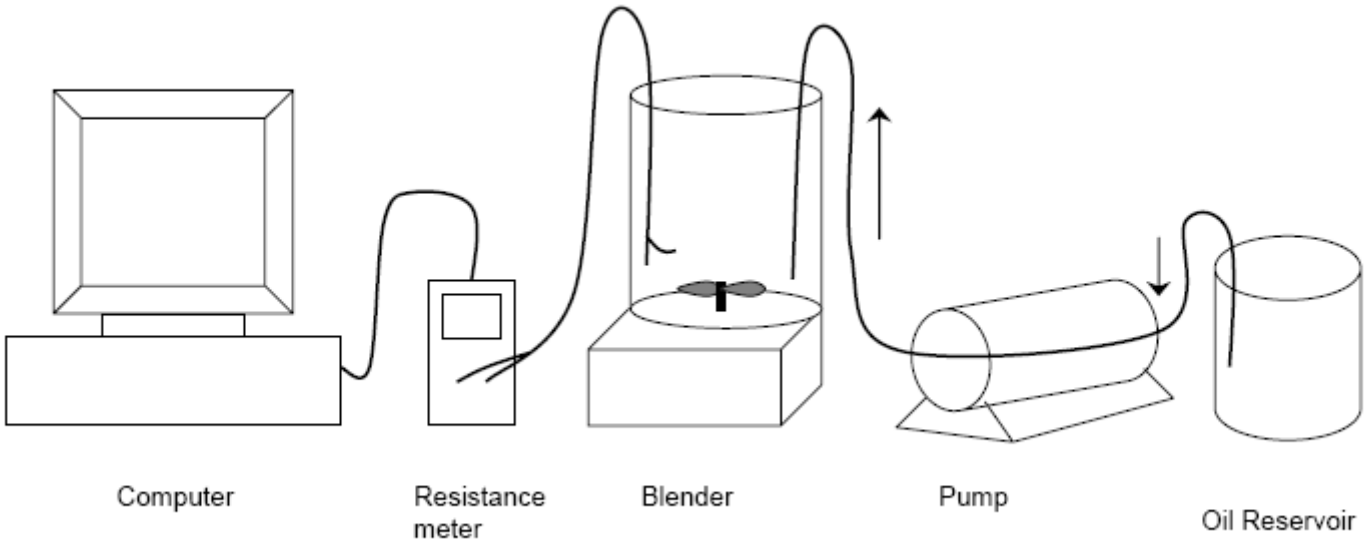

Figure 1.10. Schematic of emulsion forming and electrical conductivity measuring apparatus (Firebaugh 2002).

1.2.3.4 Destabilization Mechanisms

When the emulsifier is unable to prevent oil droplet contact, emulsions may be destabilized through flocculation and coalescence (figure 1.11) (Walstra, 2003).

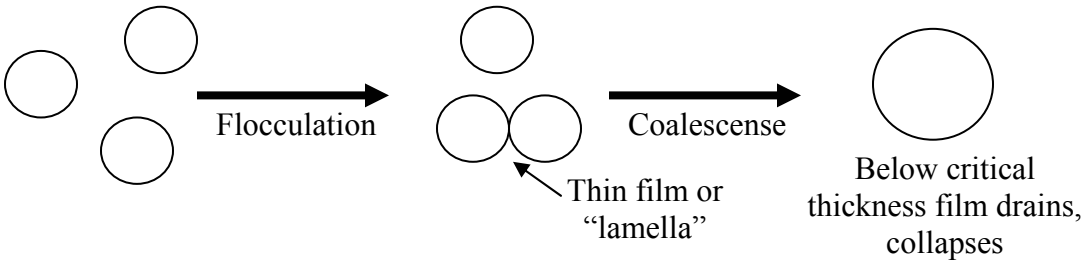

Figure 1.11. Flocculation and Coalescence.

Depletion flocculation is a mechanism where the free emulsifier in the solvent may cause destabilization. Depletion flocculation or aggregation occurs because nonabsorbing free polymers are excluded from an interface in an emulsion. The interface has a depletion layer that may overlap with another droplets depletion layer, increasing the free space in the bulk phase and increasing the entropy for the free polymer (figure 1.12) (Walstra, 1996; Walstra, 2003). Depletion flocculation has been found in soy protein emulsions with free dextran (Diftis et al., 2005).

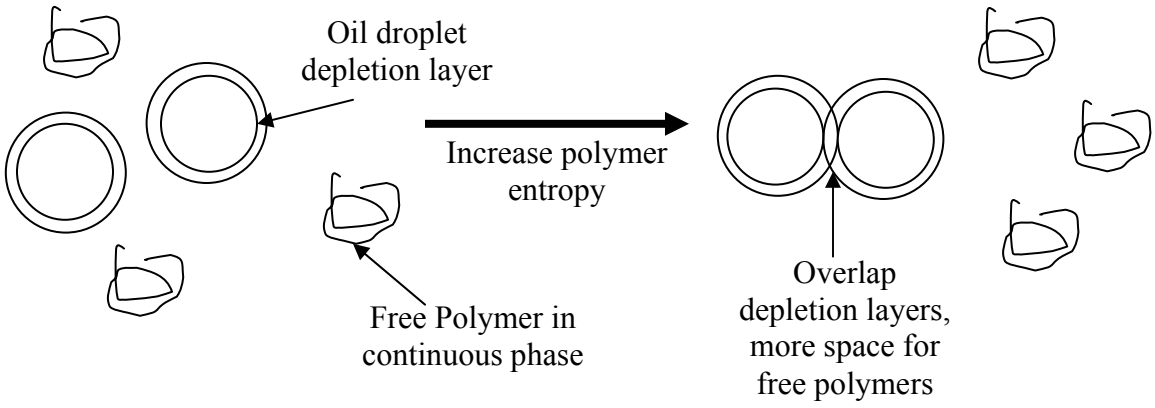

Figure 1.12. Depletion flocculation due to free polymer or particle in solution.

1.2.4.1. Functionality of Conjugates in Gelation

Soy protein isolate has previously been conjugated to reducing sugars, resulting in improvements in gelation functionality. Cabodevila et al. (1994) conjugated soy protein isolate (10-20%) to 3% xylose through the Maillard reaction using a wet heat procedure at 121°C for one hour. Conjugation was confirmed by a decrease in pH from 7.15 to 5.5 and a darkening in color. The SPI-xylose gels produced less syneresis, and had a higher elasticity resulting in a higher breaking force. The conjugation also reduced the concentration needed to form a gel. The greater elasticity of the conjugated gels was attributed to non-disulfide covalent crosslinking resulting from the Maillard reaction, because beta-mercaptoethanol-SDS had a reduced effect on the SPI-xylose gels than an SPI-glucono-delta-lactone (GDL) gel, but both gels showed similar solubility in SDS alone. The reduced effect of the beta-mercaptoethanol also suggested that the Maillard crosslinks were probably in the 11S globulin of the gel, which had more disulfides to start with, while the SDS-soluble protein was part of the 7S globulin. (Cabodevila et al., 1994).

1.2.4.2. Functionality in Emulsification: Non-Maillard Conjugates

Kato et al. (1988) conjugated dextran (75 kDa) to ovalbumin, soy protein, and lysozyme with a method utilizing cyanogen bromide instead of the Maillard reaction. The resulting ovalbumin and dextran conjugate solutions, 0.1% (w/v), demonstrated emulsifying stability and activity improvements over commercial emulsifiers (SunSoft SE-11 and Q-18S), ovalbumin and dextran mixture, dextran, and ovalbumin with 25% corn oil. Kato et al. (1988) measured additional improvements in the emulsifying properties of ovalbumin-dextran conjugates with the addition of 10% NaCl or under acidic conditions. When different proteins (soy and lysozyme) were conjugated to dextran, there were similar

improvements in emulsifying properties. However, when pectin (single-stranded chain) was conjugated to ovalbumin instead of dextran (highly branched), no improvement in emulsifying properties was measured. Heating the conjugated protein-dextran solutions to 90°C at 0.1% protein also improved the emulsifying activity. The conjugate's use as an emulsifier was based in their improved solubility in both oil and water. Overall, Kato et al. (1988) demonstrated the potential for manipulating emulsion properties of proteins through polysaccharide selection and conjugation, as well as heat and salt treatments.

1.2.4.2. Functionality in Emulsification: Maillard Conjugates

Dextran has demonstrated functionality benefits when conjugated to ovalbumin, soy protein, and gluten via the Maillard reaction (Kato et al., 1990; Kato et al., 1991; Diftis et al., 2004). Kato et al. (1990) utilized the Maillard reaction to conjugate ovalbumin with dextran. The resulting conjugates showed improvements in emulsification and solubility, just as the conjugates made with the cyanogens bromide method had shown (Kato et al., 1988; Kato et al., 1990). Preheating at 100°C increased the emulsifying properties of ovalbumin and dextran conjugates (Kato et al., 1990). The Maillard reaction was also used to conjugate pronase-treated gluten to dextran (MW 60-90 kDa), resulting in increased solubility and emulsifying capability (Kato et al., 1991).

The occurrence of conjugation of pronase-treated gluten with dextran (Kato et al., 1991), ovalbumin to dextran (Kato et al., 1990), and soy protein isolate to dextran (Diftis and Kiosseoglou, 2004) has been demonstrated with SDS-PAGE. The branched dextran conjugated to the proteins produced a dense staining pattern at the entrance to the separating gel because the large molecular weight of the conjugates prevented the protein from

migrating through the gel. The dense pattern provided support that conjugation had taken place.

Diftis et al. (2004) used the Maillard reaction to conjugate dextran (144 kDa) to soy protein isolate at a 1:1 weight ratio using a dry heat procedure at 60°C for one week. The resulting conjugate demonstrated the ability to adsorb onto oil droplet surfaces, increasing emulsion stability over non-conjugated soy proteins in systems with 10% oil and 0.5% soy protein (w/v). The conjugates enhanced emulsion stability even when partially displaced through competitive adsorption with the surface-active molecules Tween 40 and bovine serum albumin. However, glycerol monosteareate destabilized the emulsions stabilized by SPI-dextran conjugates. The destabilization was believed to be due to increased protein-protein interaction resulting in bridging flocculation.

Denaturation is often expected to decrease the functionality of proteins, including emulsification properties, due to a loss in solubility (Voutsinas et al., 1983). Soy proteins are also expected to lose solubility and precipitate near their isoelectric point, where charge is neutral (pH 4.3) (Lusas and Rhee, 1995), and so would not be useful as emulsifiers in acidic products such as salad dressing (pH 3.8) (Diftis et al., 2005). However, conjugating soy protein to dextran increases solubility, possibly because the dextran prevents the heat denatured proteins from associating (Kato et al., 1988; Dickinson and Semenova, 1992). Diftis et al. (2005) investigated the ability of SPI-dextran conjugates (dextran 144 kDa), formed through Maillard reaction, to stabilize model salad dressing emulsions. It was concluded that SPI-dextran conjugates formed by heating for one week at 60°C stabilized 50% corn oil emulsions with repulsive steric forces at a concentration of 1% protein. Conjugates formed by heating for three weeks at 60°C resulted in flocculation, attributed to

bridging of the protein between oil droplets during the longer heating time. When dextran and SPI were mixed, but not conjugated, the emulsion droplets flocculated. The destabilization was attributed to depletion effects caused by the non-adsorbing dextran (Diftis et al., 2005). On a longer time frame (100 days), however, the non-conjugated SPI-dextran mixture exhibited less emulsion destabilization than the conjugated mixture. This behavior was probably due to the more solid-like response of the non-conjugated mixture compared to the liquid-like behavior and continuous droplet rearrangement of the conjugated SPI-dextran (Diftis et al., 2005).

Emulsification ability is not entirely related to solubility. Denaturation may increase the surface hydrophobicity of a soluble protein, increasing the amphiphilic nature or the fat binding capacity. The emulsifying activity of soy protein was increased by heat denaturation at 100°C and 121°C for several minutes, correlating to a hydrophobicity increase (Voutsinas et al., 1983).

Dickinson and Semenova (1992) determined the emulsification ability of 11S globulin and BSA covalently linked to several molecular weights of dextran. It was determined that conjugating too much large hydrophilic dextran to BSA shielded the hydrophobic protein from the interface and lowered the emulsification ability of the conjugate. Also, there was an ideal ratio, different for each protein-polysaccharide combination, that provided the highest amount of conjugation and steric stabilization on the interface without having a large amount of free polysaccharide causing depletion flocculation.

1.3 Rheological Properties

Rheology is the study of the flow and deformation of matter (Steffe, 1996). The effect of each soy protein modification on viscosity may be elucidated through the use of rheological tests.

When a food gels, a continuous network of cross-linked polymers or aggregated particles (Walstra, 2003) that may contain both a viscous (fluid-like; G") and elastic (solid-like; G') component is created (Steffe, 1996). Oscillatory tests are unsteady shear measurements that subject a sample to a sinusoidal deformation in a simple shear field separating the G' and G" components. When G' is higher than G" and the lines are parallel over a range of frequencies, the material is considered to have a gel network. (Steffe, 1996)

Oscillatory tests are reliably conducted in the linear viscoelastic region, where rheological parameters are independent of stress and strain (an applied stress will produce a proportional strain). The gel network is not disturbed in the linear region, producing reliable G' and G" values. The linear viscoelastic region may be determined with a stress sweep of the material at a constant frequency. Non-linear, or fracture, properties may be determined at higher frequencies. The highest frequency that produces a linear region should be used because all smaller frequencies will contain this linear region. (Steffe, 1996)

An oscillatory shear test may be used to determine the complex viscosity (η*) of samples. The η* is calculated with G' and G" values and two viscosity values, dynamic viscosity (η') and an out of phase component of the η* (η") (Equations 2-4) (Steffe, 1996).

$$\eta' = \frac{G''}{\omega} \qquad \text{Equation 2}$$

$$\eta'' = \frac{G'}{\omega}$$ Equation 3

$$\eta^* = \sqrt{(\eta')^2 + (\eta'')^2}$$ Equation 4

The η^* is almost equal to steady shear viscosity (η) when shear rate ($\dot{\gamma}$) and frequency (ω) are equal according to the Cox-Merz rule (Equation 5) (Steffe, 1996), allowing for comparison between materials tested under oscillatory and steady shear conditions.

$$\eta^* = \eta|_{\omega=\dot{\gamma}}$$ Equation 5

1.4 Conclusions

As the complexities of soy protein continue to be elucidated the use of soy protein isolate has the potential to increase. Biochemical and rheological techniques offer the opportunity to understand the mechanisms of soy protein function in food to modify and expand on current capabilities.

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PART 2: Molecular interactions and functionality of a cold-gelling soy protein isolate

2.1 Abstract

A thermally modified, readily reconstituted, soy protein isolate (SPI), prepared at 8% protein (w/w) for three hours at 95°C, demonstrated improved heat stability and cold-set gel functionality when compared to a control SPI. When SPI was heated at 3% protein (w/w) equivalently, prior to reconstitution to 8% protein (w/w), the final viscosity was about three orders of magnitude less than the original sample. The viscosity of SPI heated at 3% protein (w/w) was still nearly two orders of magnitude less than the original sample after both samples were reheated at 8% protein. These results suggested that heat denaturation at low protein concentrations limited network formation even after the protein concentration and interaction sites increased, which in turn impacted the viscosity parameters of the system. Also, gelation was entirely prevented upon treatment of SPI with iodoacetamide, which carbaminomethylated the cysteine residues, a finding that established the role of disulfide bonds in network formation. The viscosity of the 8% protein (w/w) dispersion was also reduced by two orders of magnitude when treated with 8M urea, and when combined with 10mM DTT the viscosity of the gel was decreased by another order of magnitude. These results suggested that hydrophobic interactions or entanglements played a primary role in gel strength after disulfide bonds form. The need for a higher concentration of protein during the heating step indicated that the critical disulfide bonds are intermolecular. Based on SDS-PAGE banding patterns and the reported cysteine content of 11S subunits, it appeared that sulfhydryl/disulfide interactions could be attributed to the 11S basic subunits, while 7S α', α, and β participated in hydrophobic interactions or entanglements. Ultimately, the functionality produced by these combined protein-protein interactions produced a powdered soy protein isolate ingredient with consistent cold-set and thermal gelation properties.

2.2 Introduction

Soy protein isolate (SPI) is a high quality plant protein source, generally greater than 90% protein, with associated nutritional benefits that have increased its utilization to the food industry in recent years. Soy protein is one of several heat-gelling proteins. Recently cold set gelation of soy protein gels has been demonstrated using a preheating step to denature the proteins, followed by addition of calcium to induce gelation through electrostatic interactions (Maltais et al., 2005). This example of cold gelation demonstrated the potential for modifications to SPI to improve and expand current functional capabilities. An understanding of soy protein and current gelation mechanisms is required to increase SPI functionality through modification

Soy protein isolate consists of two major storage proteins, β-conglycinin (7S) and glycinin (11S), that vary in amount depending on the soybean variety. Their amino acid composition, molecular weights, and physical properties provide different mechanisms for structure formation and functionality. The molecular weight (MW) of the 7S fraction ranges from 140-210 kD, containing 3 major subunits, α', α, and β, with molecular weights of 58 kD, 57 kD, and 42 kD, respectively (Thanh and Shibasaki, 1977; Thanh and Shibasaki, 1978; Koshiyama, 1968). The MW of the 11S fraction is approximately 320-375 kD, containing six acidic (MW 35-38 kD) and six basic (MW 18-20 kD) disulfide-bonded subunits (Utsumi et al., 1981; Badley et al., 1975).

Molecular interactions between these subunits provide potential for developing alternative functional properties. The subunits that aggregate and become insoluble during heating contribute to gelation (Wolf, 1970; Petrucelli and Anon, 1995a.; Petrucelli and Anon, 1995b.; Sorgentini et al., 1995). The α', α, and β subunits have been observed to aggregate

and become insoluble above certain protein concentrations during heating (Sorgentini et al., 1995; Utsumi et al., 1984). The basic subunit has also been shown to become insoluble during heating (Sorgentini, et al. 1995; Wolf, 1970; Utsumi et al., 1984). The nature of these aggregates and a greater understanding of soy protein gels is a continuous subject for research.

Soy protein isolate has been shown to form a gel during heating above a concentration of 8% powder w/w (Circle et al., 1964; Sorgentini et al., 1995; Catsimpoolas and Meyer, 1970). Previous insight into soy protein isolate structure involved various chemical reagents: cysteine (Circle et al., 1964; Wang and Damodaran, 1990), β-mercaptoethanol (β-ME) (Briggs and Wolf, 1957; Catsimpoolas and Meyer, 1970; Utsumi et al., 1984; Wolf, 1993), dithiothreitol (DTT) (Wolf, 1993; McKlem, 2002), N-ethylmaleimide (NEM) (Briggs and Wolf, 1957; Catsimpoolas and Meyer, 1970; Shimada and Cheftel, 1988; Wang and Damodaran, 1990), iodoacetamide (Wolf, 1993), sodium borohydride ($NaBH_4$) (Wolf, 1993), sodium thiocyanate (NaSCN) (Nagano et al., 1994), sodium sulfate (Na_2SO_3) (Petrucelli and Anon, 1995b), urea (Catsimpoolas et al., 1969; Petrucelli and Anon, 1995b), sodium dodecyl sulfate (SDS) (Petrucelli and Anon, 1995b), and guanidine hydrochloride (GHCl) (Nagano et al., 1994). Use of these reagents revealed the existence of sulfhydryl/disulfide interchange, hydrogen bonds, electrostatic, and hydrophobic interactions in thermally induced soy protein gels as mechanisms for soy protein gelation. The presence of disulfide bonds and sulfhydryl groups in soy protein has been previously investigated, indicating most opportunities for disulfide bonding are in the 11S fraction (Shimada and Cheftel, 1988; Thanh and Shibasaki, 1977; Koshiyama, 1971). Sulfhydryl groups provide the opportunity for irreversible covalent interactions. The 7S fraction contains four

sulfhydryl groups that participate in two intramolecular disulfide bonds, whereas 11S has 48 sulfhydryl groups, many of which interact in bonds between the acidic and basic subunits (Koshiyama, 1971; Hermansson, 1978).

Although most researchers agree that sulfhydryl / disulfide interchange plays a part in gelation due to the decrease in gel strength upon the addition of disulfide reducing agents, their actual role with respect to other molecular interactions has not been fully established and may depend on gelation temperature and protein concentration. Circle et al. (1964) observed that soy protein gels are irreversible under heating conditions of 100°C for 30 minutes and concluded that disulfide bonds must be a part of the gelation mechanism. Alternatively, Catsimpoolas and Meyer (1970) proposed a schematic involving a sol, progel, and gel, where the gel, formed at 80°C, was reversible and therefore must be formed through noncovalent interactions. In addition, Catsimpoolas and Meyer (1970) found that small amounts of reducing agents reduced gel strength, but large amounts allowed the gel to unfold and create more preferential interactions. However, further support for disulfide bonds was demonstrated when SPI gels were shown to increase in firmness with temperature up to 120°C, coincident with a decrease in free sulfhydryl groups and protein solubility (Shimada and Cheftel, 1988).

Further debate as to the role of disulfides was provided when McKlem (2002) revealed that initially disulfides are important, but become less significant post thermal treatment, once hydrophobic interactions occur. Alternatively, Wang and Damodaran (1990) reported that the increase in molecular weight due to intermolecular disulfide bond formation resulted in gelation by creating a network capable of entanglement. Utsumi and Kinsella (1985) demonstrated the presence of both hydrogen bonds and disulfide bonds in SPI gels.

The presence of hydrogen bonds was also supported through thermoreversibility of soy protein gels (Nagano, 1994; McKlem, 2002). Hydrophobic interactions have been shown to be important at lower gelation temperatures (80°C), where only the sulfur deficient 7S group is denatured (Catsimpoolas and Meyer, 1970).

The objective of this work was to utilize soy protein concentration and thermal denaturation to produce a soy protein isolate that readily reconstitutes and gels at ambient temperatures upon the addition of water. Insight into the functional mechanisms of cold gelation, depending on protein concentration at denaturation, is provided. An understanding of the functional mechanism that provided the versatility of this SPI ingredient enables further expansion of soy protein-based ingredients.

2.3 Materials and Methods

2.3.1 Chemicals

Urea, dithiothrietol (DTT), iodoacetamide, β-mercaptoethanol (β-ME), hexane, Trizma base, sodium dodecyl sulfate (SDS), glycine, ethylenediaminetetraacetic acid (EDTA), Bis-Acrylamide (40%), glycerol, bromphenol blue, ammonium persulfate, methanol, acetic acid, and sucrose were purchased from Sigma Chemical (St. Louis, MO). All other chemicals were of analytical grade. Distilled water or purified Milli-Q water (Millipore Corp., Billerica, MA) of 18 mΩ-cm resistivity was used for the preparation of all analytical reagents and buffers.

2.3.2 Extraction of Soy Protein Isolate

Soy protein isolate (SPI) was prepared from soybeans (*Glycine max L. Merr., cv.* Brim). Soybean seeds were ground in a Retsch centrifugal grinder, Model ZM100 (Newtown, PA), equipped with a 24-tooth rotor and a 1.0-mm stainless steel ring using a

motor speed set at 14,000 rpm. This setting produced ground samples with a uniform particle size of less than 0.5 mm for efficient oil and protein extraction. Oil was extracted from the ground meal using a Soxhlet apparatus with warm hexane. Native protein was extracted batchwise from the defatted meal using the commercial procedure described by Lusas and Rhee (1995) and brought to pH 6.8 with NaOH (figure 2.1). The final protein concentration was approximately 3% at pH 6.8. The majority of the protein solution was lyophilized and used as a control while the remainder was modified as described herein. The lyophilized SPI was ground in a centrifugal grinder, but with a 0.5mm sieve size, to produce SPI in a powdered form. The protein content of the lyophilized SPI was determined by the Dumas combustion method (Nielsen, 1998). Unless otherwise stated, all experiments and procedures were performed at room temperature.

2.3.3 Protein Modification

The lyophilized SPI was rehydrated to 8% protein (w/w) (figure 2.2). A portion of the 8% solution was lyophilized again in the native state, serving as the control (native SPI). The remaining 8% solution and the remaining portion of the native protein still in solution at ~3% protein (w/w) was heated for 3 hours at 95°C ± 3°C and lyophilized. Both samples were ground to powdered form as previously described and stored at 4°C. The heated samples are referred to as 8% SPI and 3% SPI.

2.3.4 Sample Preparation for Viscoelastic Tests

The 8% SPI and the native SPI were prepared to 8% protein (w/w) using either distilled water, 8M urea, 10mM DTT, or 8M urea + 10mM DTT. The 3% SPI was also prepared to 8% protein (w/w) in distilled water. All samples were allowed to rehydrate overnight and tests were performed within 48 hours of sample preparation.

2.3.5 Measurement of Viscoelastic Properties

A Rheologica Stresstech Rheometer, ATS Rheosystems (Bordentown, NJ), was used to measure viscoelastic properties of all test solutions as a function of frequency (0.005 – 10Hz) and temperature (from 10°C to 90°C) within a predetermined linear viscoelastic region. Thermal measurements began at room temperature (25°C), and were continuously recorded as the samples were cooled to 10°C and then heated to 90°C (2.5°C per minute). The sample temperature was held at 90°C for 30 minutes, simulating potential processing conditions. The samples were cooled to a final temperature of 25°C (2.5°C per minute), establishing a baseline comparison for sample temperature stability from the beginning of the test to the end. Pressure was applied to the sample using an ATS Rheosystems sealed cell (Bordentown, NJ) to prevent moisture loss, and data were recorded in triplicate.

2.3.6 Polyacrylamide Gel Electrophoresis

The subunit profile of soluble and insoluble soy protein fractions was evaluated using a Bio-Rad (Richmond, CA) Protean II vertical slab gel apparatus according to Chua (1980) with the following modifications. A soybean reference protein (SRP) was prepared according to the procedure of Kwanyuen and Wilson (2000). The SRP was extracted from full-fat soybean meal for 30 minutes at a 1:20 (w/v) ratio with 0.03 M Tris-HCl buffer, pH 8.0 that contained 0.1 M β-mercaptoethanol. The SRP was then centrifuged at 10,000 xg for 10 minutes in an Eppendorf centrifuge Model 5417 C (Hamburg, Germany). The native SPI, 3% SPI, and 8% SPI samples were rehydrated to 8% protein (w/w) and centrifuged at 5,200 xg for 10 minutes to separate the soluble and insoluble fractions. The supernatant containing the soluble protein was pipetted into a separate container and the pellets were resuspended to 2 ml in water. Protein concentrations of the soluble and insoluble fractions were determined

according to Bradford (1976). Each sample contained about 25 mg/ml protein. The three SPI samples and SRP were mixed at a 1:1 ratio with 0.06M Tris-HCl, pH 8.0, 5% SDS prepared with 0.1M β-mercaptoethanol. In some samples, 0.1M β-mercaptoethanol was excluded in order to evaluate the disulfide banding pattern. Proteins were dissociated in a boiling water bath for 10 minutes. The tracking dye was added to the samples at a concentration of 10% glycerol and 0.025% bromophenol blue. Samples were loaded onto the gel (10 μg protein per well) and electrophoresed using a 10% to 20% gradient polyacrylamide gel. Blank wells were left in-between loaded samples to prevent cross-contamination during migration and to facilitate accurate quantification with densitometry. A constant current of 10mA/gel was used until tracking dye reached the bottom of the gel.

Gels were fixed with 40% (v/v) methanol and 10% (v/v) acetic acid for an hour on an orbit shaker and then stained overnight in 0.25% (w/w) Coomassie Brilliant Blue R250, 40% (v/v) methanol, and 10% (v/v) acetic acid on the orbit shaker. Gels were destained in 40% (v/v) methanol and 10% (v/v) acetic acid. The destaining solution was changed when it reached a similar color to the gel. After at least three changes of the destaining solution, gels were soaked in distilled water for 15 minutes, scanned with a Molecular Dynamics Personal Densitometer SI (Sunnyvale, CA), and analyzed with Molecular Dynamics ImageQuant software. Volume integration was used in data analysis to determine the total absorbance of entire protein bands. The apparent absorbance of each protein subunit was obtained by subtracting the local average background absorbance from the total absorbance of the protein subunit within the same gel volume. Gels were sandwiched between cellophane and dried in a Bio-Rad GelAir dryer.

2.3.7 Differential Scanning Calorimetry (DSC)

Thermal denaturation of the soy proteins was assessed with a Perkin-Elmer DSC 7 (Wellesley, MA), calibrated with indium and mercury using N_2 purge gas. Native SPI and 3% SPI were rehydrated to 20% protein (w/w) and analyzed in a hermetically sealed stainless steel pan with an empty pan of similar weight as the reference. The samples were heated from 10-110°C at 10°C/min, held at 110°C for 1 min, cooled to 10°C at 10°C/min, held at 10°C for 1 min, and heated to 110°C at 10°C/min. Denaturation temperatures were determined at peak height and enthalpy was determined from area under the peak for the 7S and 11S protein fractions.

2.3.8 Determination of Sulfhydryl Content

Sulfhydryl content was measured according to the method of Shimada and Cheftel (1988) with modifications. Native SPI and 8% SPI were solubilized at 4mg/ml in 0.086M Tris-HCl at pH 8.0 containing 0.09M glycine, 4mM Na_2EDTA, 0.5% SDS, 6M urea, and 10mM dithiothreitol (DTT). The disulfide reduction was carried out at room temperature under vacuum for one hour and excess DTT was then removed by gel filtration using Sephadex G-25 column chromatography previously equilibrated with 0.086M Tris-HCl at pH 8.0 containing 0.09M glycine and 4mM EDTA. The sulfhydryl content was determined according to the procedure of Ellman (1959) using 10mM DTNB in 0.1M NaH_2PO_4 at pH 8.0. A Shimadzu 2101 UV-VIS spectrophotometer (Tokyo, Japan) was used to quantify the free sulfhydryl content at wavelength 412nm.

Protein content was determined after gel filtration using the procedure of Lowry et al. (1951) as modified by Bensadoun and Weinstein (1976), a procedure involving precipitation of the protein from solution to remove interfering chemicals prior to Lowry analysis. The

protein solution was sampled at 0.2, 0.4, 0.6, 0.8 and 1.0 ml and diluted in 3 ml of water. Twenty-five µl of sodium deoxycholate was added to each protein sample and reacted for 15 minutes. The protein was precipitated by addition of 1ml of 24% trichloroacetic acid and collected upon centrifugation at 3,300 xg for 30 minutes. The protein pellets were redissolved in 1.5 ml Lowry Reagent C (50 parts of Reagent A (2% Na_2CO_3 in 0.10N NaOH) + 1 part of Reagent B (0.5% $CuSO_4 \cdot 5H_2O$ in 1% sodium tartrate)). After 10 minutes of incubation, 0.15ml of 1N Folin-Ciocalteu reagent was added, and the solutions reacted for 45 minutes in the dark. Bovine serum albumin was used to generate a standard curve. Absorbance was measured on the Shimadzu spectrophotometer at wavelength 730nm.

2.3.9 Carbaminomethylation of SPI

Native SPI (lyophilized only once after isolation) was reacted with iodoacetamide according to the method of Aitken and Learmonth (1996). Protein solutions (8% w/w) were dissolved in 0.6M Tris-HCl, pH 8.6 containing 8M urea. Disulfide bonds were reduced by adding 5M DTT at 0.166% of the total weight. The protein solution was sealed, evacuated, and flushed with nitrogen and held for three hours to fully reduce the disulfide bonds while preventing air oxidation of sulfhydryl groups to disulfide bonds. The sulfhydryl groups were then covalently linked to iodoacetamide, which was added as 10% of the total weight, using a concentration of 500mM. Carbaminomethylation proceeded in darkness at 38°C for 30 minutes under anaerobic conditions, as described previously. The solution was dialyzed against water for 40 hours with three changes of water. The protein sample was then lyophilized and ground to a powder in the same manner as previously described. The protein content was determined to be 98.2% by the Dumas combustion method (Nielsen, 1998). The sample was prepared at 8% protein (w/w), heated for 3 hours at 95°C ± 3°C, lyophilized, and

then ground to a powder in the same manner as previously described. A control was prepared without a thermal treatment. The powders were rehydrated to 8% protein w/w and visual observations were recorded.

2.4 Results and Discussion

2.4.1 Viscoelastic Response in Water and Urea

A critical concentration for heat induced gelation of soy proteins was previously reported to be approximately 8% (w/w) SPI powder (Sorgentini et al., 1995; Circle et al., 1964). During laboratory isolation, the soy protein concentration was around 3%. In this work, the isolated soy protein solution was freeze dried and rehydrated at 8% protein (w/w) before thermally inducing gelation, then dried to a powder (figure 2.2). In a commercial setting, proteins may become completely denatured during isolation (Hermansson, 1978; Wagner et al., 1992). Commercially prepared soy protein isolates have been shown to provide different functionality and mechanisms of gelation depending on degree of denaturation, method of processing, or other unknown causes (Hermansson, 1978; Hermansson, 1986; Chronakis, 1996). Wagner et al. (1992) suggested that the denatured soy protein formed insoluble aggregates that would not interact in new structures. Although the protein concentration at denaturation of fully denatured commercial isolates is not known, this study was also conducted at 3% protein (w/w) based on laboratory data to determine the functionality of a protein already subjected to heat denaturation at low concentration. The SPI powders were determined to be 87.6% protein by the Dumas combustion method (Nielsen, 1998). Although thermal treatments occurred at different protein concentrations, used to label the samples, all samples were on an equivalent 8% (w/w) protein basis for testing.

The complex viscosity (η*) of the native SPI, the 3% SPI, and the 8% SPI are shown in figure 2.3a. The native and 3% SPI displayed similar viscosities through varying oscillatory speeds. The η* for the 8% SPI was higher than the 3% SPI and native SPI at low frequencies, but decreased in viscosity at higher frequencies. The 3% SPI and native SPI did not display the same viscosity behavior as the 8% SPI because these isolates already exhibited a lower viscosity, indicating less structure. After heating, the native SPI, 3% SPI, and 8% SPI all demonstrate the elastic properties of a gel, but the 3% SPI never achieved the strength of the gels initially heated at 8% protein (w/w) (figure 2.3b).

The viscoelastic response of the native SPI and 8% SPI with respect to temperature is shown in figures 2.4a and 2.4b. The 8% SPI demonstrated cold-gelling ability immediately upon hydration at 25°C and stability throughout heating and cooling (figure 2.4b), whereas the native isolate hydrated at the same protein content did not achieve this functionality until it was heated to 90°C for 30 minutes (figure 2.4a). The 3% SPI never reached the same level of η* as the native SPI and 8% SPI (data not shown). The stability of the 8% SPI viscosity through a heating-cooling cycle demonstrated elastic behavior typical of a gel.

Urea has previously shown interference with SPI gelation, demonstrating the presence of hydrophobic interactions in traditional SPI gelation (Catsimpoolas et al., 1969; Catsimpoolas and Meyer, 1970; McKlem, 2002). In this work, urea prevented interactions within the native isolate, and a viscosity increase did not occur during heating (figure 2.4a). The response of the native isolate upon the addition of urea indicated that hydrophobic interactions were primarily responsible for the viscosity increase. Urea interrupted the hydrophobic interactions in the 8% SPI to a viscosity level more similar to the native SPI,

demonstrating the contribution of hydrophobic interactions to the cold-gelling properties of the SPI ingredient (figures 2.4a and 2.4b).

The importance of hydrogen bonding was also supported by the presence of thermally reversible activity for the native and 8% SPI. The increase in viscosity when cooled to 10°C or 25°C and the decrease in viscosity when heated to 90°C, both with and without urea, supported the notion that hydrogen bonding increased the final viscosity of the cooled product. Hydrogen bonds were previously reported to be involved in 7S gel formation by Nagano et al. (1994).

While the data support hydrophobic interactions as a primary interaction responsible for soy protein gel strength, work by Sorgentini et al. (1995) indicated that after heating at low concentrations, the protein remained denatured in the soluble state with hydrophobic sites exposed. The insoluble state at low concentrations had a low surface hydrophobicity, indicating that hydrophobic interactions led to aggregation and insolubility. Therefore, after heating at a concentration of ~3% protein (w/w), the denatured soluble protein should be able to interact when the protein is rehydrated at a higher concentration, aggregate, and become insoluble. However, figure 2.3 reveals that rehydrating protein above the critical concentration (8%) that was already denatured below the critical protein concentration (3%) will not produce the same viscosity increase as protein denatured above the critical concentration. Even when the 3% SPI was reheated at the higher concentration, it did not reach the viscosity of protein solutions originally heated at the higher concentration. The inability to reach a higher viscosity suggests irreversible denaturation of the 3% SPI, which prevents the formation of more functional interactions under more favorable conditions, i.e. higher concentration, even if the hydrophobic groups are exposed as described by Sorgentini

et al. (1995). This idea is supported by Wagner et al. (1992), who found that soy protein isolates denatured during commercial isolation formed aggregates that would not interact in new structures.

2.4.2 Denaturation of the Proteins Determined Through DSC

Differential scanning calorimetry (DSC) was employed to determine the extent of protein denaturation of native SPI and 3% SPI (figure 2.5). The native SPI shows a peak where 11S is denatured around 95°C. This peak disappears when the native SPI is cooled and heated a second time, providing a baseline for SPI denaturation. The 3% SPI was fully denatured during the first heating curve, demonstrating that protein heated below critical concentration denatures, potentially exposing hydrophobic sites as indicated in the work of Sorgentini et al. (1995). The lower viscosity of the 3% SPI in comparison to the 8% SPI, even after reheating at 8% protein, further supports the idea that 3% SPI denatures when heated, irreversibly aggregating into a conformation less favorable to gelation mechanisms. The irreversible behavior of 3% SPI suggests that while hydrophobic interactions are important, covalent interactions that form during denaturation impact the protein functionality. Otherwise, the 3% SPI would form stronger interactions with exposed hydrophobic groups upon reheating. The idea that hydrophobic interactions do not produce gel strength alone is further supported by Wang and Damodaran (1990), who state that a self-supporting protein gel with thermal and mechanical stability is dependent on entanglements. The entanglements are dependent on protein concentration and molecular weight. In this event the true role of disulfide bond formation was to increase the molecular weight of the proteins above a critical molecular weight, enabling gelation. The prerequisite of a critical molecular weight may deem intermolecular disulfide bond formation essential to soy protein

gelation. The idea that disulfide bonds are essential to soy protein gelation has been supported by others based on the observation of the loss of gelation with the addition of reducing agents (Shimada and Cheftel, 1988; Circle et al., 1964; Petrucelli and Anon, 1995b).

2.4.3 Viscoelastic Response in DTT and Urea + DTT

The role of disulfide bond formation in the native SPI and 8% SPI was further investigated with the reducing agent DTT. The addition of DTT reduced the viscosity of both the native SPI and 8% SPI (figures 2.4a and 2.4b), but this effect was seen more in the native SPI (figure 2.4a). The presence of DTT diminished the η^* of the native SPI ten fold upon initial rehydration at 25°C and cooling to 10°C. This η^* reduction may be due to breakage of disulfide bonds present in the native SPI, indicating importance of these bonds initially. It would appear, as proposed by McKlem (2002), that disulfides are not important after heating to 90°C, as the native SPI containing DTT increased in viscosity with heating to the same point as the native SPI without DTT. However, the optimum temperature for DTT reaction is around 20°C at a neutral pH and the half-life of DTT decreased nearly 10 fold with every 20°C increase in temperature (Stevens et al., 1983). Therefore, DTT likely maintained disulfide reduction of the protein initially but decreased in stability as temperature increased to 90°C, allowing disulfide bonds to form again.

When urea and DTT were used in combination and added to the 8% SPI solutions, the cumulative decrease in viscosity was 10 times greater than with urea alone (figure 2.4b). The viscosity was relatively similar for both native SPI and 8% SPI when urea and DTT were used jointly (figures 2.4a and 2.4b), indicating that DTT reduced disulfide bonds that were initially in the protein but inaccessible until urea was present to denature the protein and

permit DTT access to internal bonds. When the DTT was deactivated upon heating, the urea likely prevented protein interactions that would allow new disulfide bonds to form.

Disulfide/sulfhydryl interchange during heating would create intermolecular bonds between the proteins from the intramolecular disulfide bonds and free sulfhydryl groups currently present. Disulfide/sulfhydryl interchange has been previously proposed by Shimada and Cheftel (1988) who found approximately 6 μMol sulfhydryls / g protein. In this work, the soy protein isolate was found to contain 0.20-0.35 μMol sulfhydryls / g protein in the reduced state, but the difference in sulfhydryl content may be attributed to cultivar differences. According to figures 2.4a and 2.4b, the disulfide bonds do not contribute as much to viscosity as hydrophobic interactions, based on the viscosity difference upon the addition of urea as opposed to urea+DTT. However, intermolecular disulfide bond formation may explain the critical concentration requirement. Sulfhydryl/disulfide interchange would require the heating step to occur at a concentration that permits a sufficient amount of protein interaction and the overall number of disulfide bonds would not necessarily change.

2.4.4 Effect of Iodoacetamide, Urea, and DTT in Combination on Gelation

While DTT primarily reduces disulfide bonds and becomes inactivated over time, especially upon exposure to high temperature, iodoacetamide covalently complexes with sulfhydryl groups, preventing disulfide formation over time. Therefore, iodoacetamide was reacted with native soy protein in the presence of DTT and urea to reduce disulfide bonds and prevent reformation. The presence of urea permitted DTT access to internal disulfide bonds, and reduction to free sulfhydryl groups took place under optimum time and temperature conditions. While the sulfhydryl groups were in the reduced state, the iodoacetamide covalently bonded with them, preventing reformation of disulfide bonds. The

absence of disulfide bonds produced an SPI with a viscosity similar to water, even after heat treatment for three hours at 95°C ± 3°C. The reduced solution began to phase separate immediately without agitation (Figure 2.6), suggesting that intermolecular disulfide bonds are required for SPI gelation functionality, and other interactions are dependent on the initial presence and viscosity increase produced by disulfide bonds. The need for intermolecular disulfide bonds provides support for Wang and Damodaran (1990) who demonstrate that gelation is based on entanglements at a critical molecular weight.

2.4.5 Further Support for Intermolecular Disulfides with SDS-PAGE

Soy protein gels have been considered both reversible and irreversible, largely depending on the gelation conditions, such as temperature and protein concentration, involved. The presence of intramolecular or intermolecular sulfhydryl groups may assist in explaining the temperature-dependent reversible nature of soy protein gels. The SDS-PAGE was undertaken in the presence and absence of β-ME and banding patterns were examined for the soluble and insoluble portions of the native SPI, 3% SPI, and 8% SPI. There are only small numbers of cysteine in the subunits of 7S (Koshiyama, 1971; Thanh and Shibasaki, 1977). Therefore, the 11S would be essential for the disulfide bonding effect, while the 7S and 11S subunits would be expected to associate noncovalently (Petruccelli and Anon, 1995b).

Concentration and extent of denaturation each influence the amount of insoluble protein present in soy protein isolate, and subsequently the viscosity. The DSC results have shown that the 7S component denatured at a much lower temperature (Tmax = 75°C) than 11S (Tmax = 95°C) (data not shown). Earlier work has shown that these values range from 68-82°C for 7S and 83-95°C for 11S (Sorgentini et. al., 1995; German et al., 1982; McKlem,

2002). These differences in denaturation temperatures may be attributed to protein fraction variations between cultivars. Sorgentini et. al. (1995) determined that heating aqueous dispersions at concentrations greater than 8% SPI powder (w/w) caused increasing amounts of protein to aggregate and become insoluble. At 80°C, mainly 7S protein subunits are capable of denaturation and aggregation. At 100°C, both 7S and 11S subunits may denature and subsequently aggregate. The amount of insoluble protein increased quickly, probably as a result of enhanced protein contact resulting in aggregation above 8% protein (w/w). The work of Sorgentini et. al. (1995) also supported Wang and Damodaran's theory that once a critical molecular weight is reached the proteins may entangle and gel.

The SDS-PAGE electrophoresis performed in the presence and absence of β-ME was employed to determine which subunits were present in the soluble fraction versus the insoluble fraction of thermally induced soy protein gels. Figures 2.7 and 2.8 support the hypothesis that intermolecular disulfide bonding influenced the subunit concentration of each fraction. Densitometry results showed an 11S to 7S ratio of 2.1, indicating the potential for disulfide bonding with the large amount of 11S present. Figure 2.7a and 2.7b show the protein banding profiles of native SPI, 3% SPI, and 8% SPI with soluble and insoluble fractions separated. The 8% SPI lost most of its soluble portion to the insoluble portion, probably due to a high degree of denaturation and a concentration capable of initiating aggregation. The native SPI profile is similar to the 3% SPI, except for a few heat sensitive disulfide bonded bands that are no longer present in the 3% SPI profile, such as the 11S acidic and basic disulfide bonded bands (Wolf, 1993). Large molecular weight proteins were present in all of the fractions in the absence of β-ME and remained on top of the resolving gel. The large molecular weight proteins were reduced upon the addition of β-ME, indicating

the presence of disulfide bonds in these polymers. Similar electrophoretic results were observed by Wang and Damodaran (1990). The fractions without β-ME contained large molecular weight polymers that streaked the gels, and were therefore filtered. The insoluble 8% SPI was too thick to filter, indicating that intermolecular disulfide bonds had most likely formed through protein contact during heating, resulting in large molecular weight polymers. The insoluble percentages for 8% SPI in figure 2.8b may be low because subunits were too large to migrate through the gel.

The SDS-PAGE revealed that the increase in aggregated and insoluble protein of the 8% SPI was a combination of the basic subunit, and the α', α, and β subunits, which disappeared from the soluble fraction (figure 2.7a and 2.7b). The loss of these subunits to the insoluble fraction was also seen by Sorgentini et al. (1995) at high protein concentrations. Wolf (1970) observed the aggregation and loss of the basic subunit to the insoluble fraction during heating. Utsumi et al. (1984) determined a decrease in the basic subunit and the β subunit in the soluble fraction at 0.5% protein concentration. The acidic subunit content increases in the soluble fraction when β-ME is present (figure 2.8a), likely due to breaking of the acidic and basic disulfide bonds and noncovalent links during heating (Wolf, 1993; Sorgentini et al., 1995). The change of the basic subunit from the soluble to the insoluble fraction above the critical concentration would prevent acidic/basic interactions from reforming. When β-ME was not present, the acidic and basic groups were similar in amount for all except soluble 8% SPI, which had a high acidic subunit content (figure 2.8b). The heat may break the acidic and basic unit in this case (Wolf, 1993).

The progression of interactions producing the 8% SPI functionality is summarized in figure 2.9. A critical concentration is necessary to allow protein contact and intermolecular

disulfide bond formation until the protein is in a molecular weight range conducive to entanglements or hydrophobic interactions so that a gel network is formed (Path A). Heating below this concentration did not facilitate protein-protein interactions, and the protein irreversibly denatured without forming a gel network (Path B). The use of DTT and urea allowed the protein to unfold and disulfide bonds to be reduced, while iodoacetamide covalently linked to the reduced sulfhydryl groups. When these reagents (DTT, urea, and iodoacetamide) were used, no viscosity increase was observed, indicating a lack of protein-protein interaction (Path C). After urea was dialyzed out of the solution, hydrophobic interactions would be expected to occur during heating if they were an important part of the molecular interactions in soy protein gelation. The lack of any viscosity in the iodoacetamide reacted solution lends support to the idea that intermolecular disulfide bonds are the primary gelation mechanism of the cold-gelling soy protein isolate ingredient.

2.5 Conclusion

The sample preparation for the 8% SPI included a second freeze drying step subsequent to heating for 3 hours at 95°C ± 3°C to produce a convenient powdered ingredient. The temperature treatment of the 8% SPI eliminates the need for a heat step during product processing and allows the ingredient to be used in heat sensitive products. The 8% SPI has been observed to disperse quickly and show an immediate increase in viscosity in water at room temperature compared to the native SPI. The 8% SPI also maintains a stable viscosity through heating and cooling cycles. The 3% SPI did not show a viscosity increase compared to the native SPI and displayed significant changes during heating and cooling. The 3% SPI remained lower in viscosity than the native SPI and the 8% SPI after all samples were subjected to a thermal treatment to 90°C at 8% protein (w/w).

Differential scanning calorimetry showed that 3% SPI is denatured when thermally treated to 95°C, indicating that the proteins irreversibly denature to a form incapable of establishing the required interactions necessary for increased functionality, even upon reheating at a higher protein concentration.

The use of urea, DTT, and iodoacetamide decreased the viscosity of SPI dispersions. The effect of the reagents on soy protein solutions supports the hypothesis that cold gelation functionality is produced through a combination of intermolecular disulfide bond formation, hydrophobic interactions, and hydrogen bonds. Disulfide interactions seem to be necessary for subsequent noncovalent interactions to occur. Therefore a protein concentration capable of inducing sulfhydryl/disulfide contact is required initially.

2.6 Works Cited

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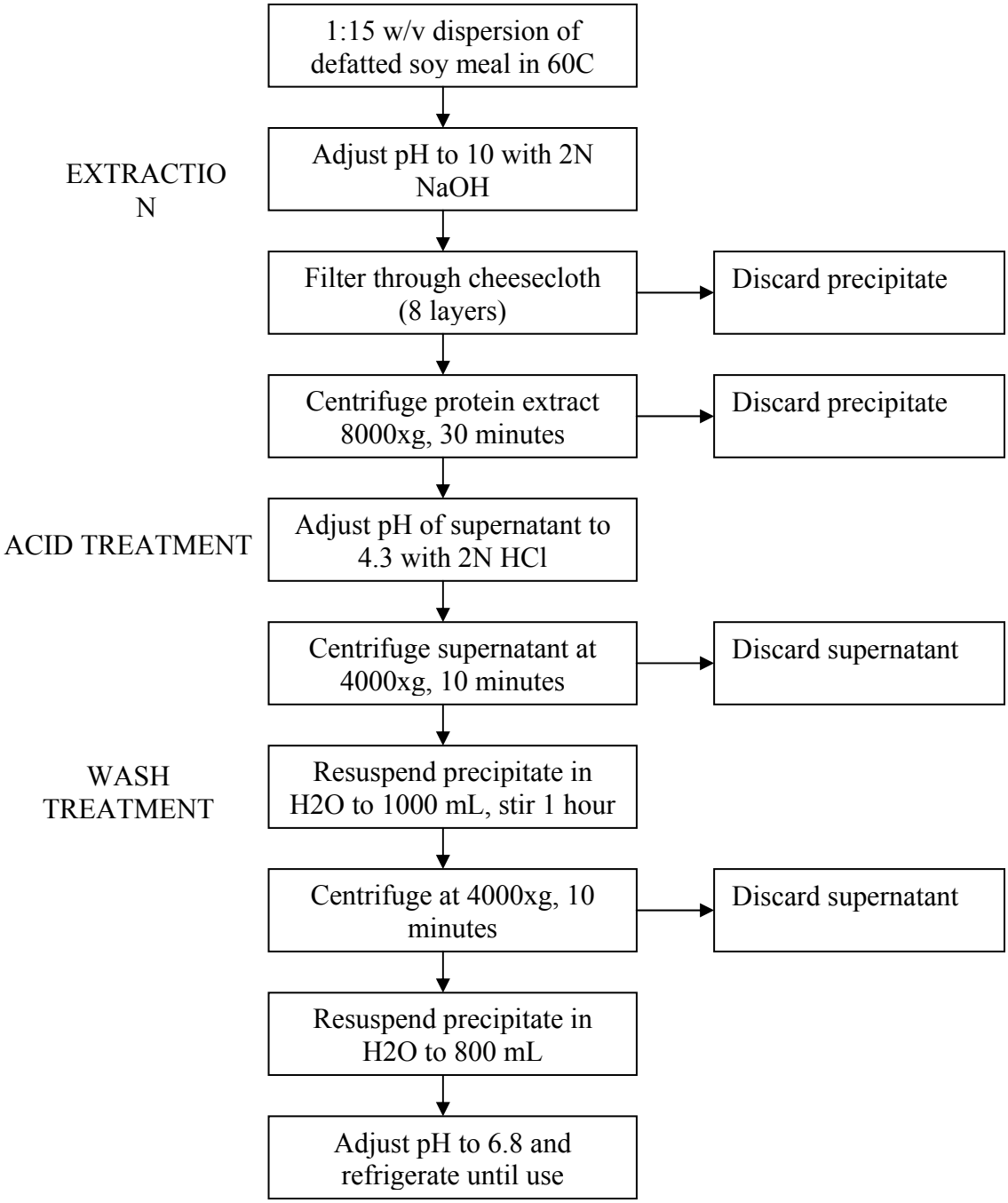


Figure 2.1. Flow Chart of Soy Protein Isolate Preparation.

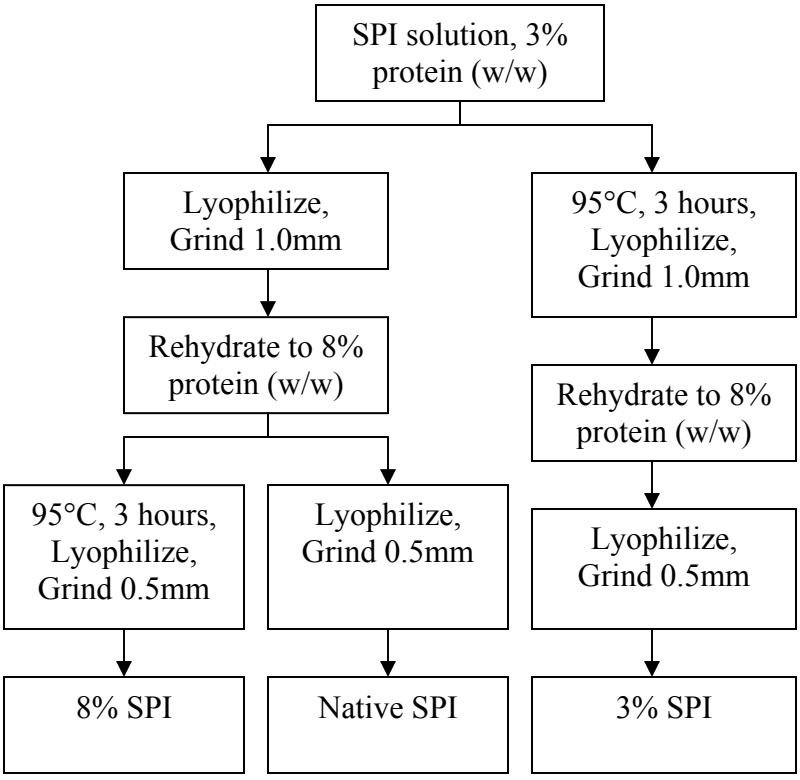


Figure 2.2. Preparation of soy protein isolate samples. All samples were rehydrated to 8% protein (w/w) for viscoelastic testing.

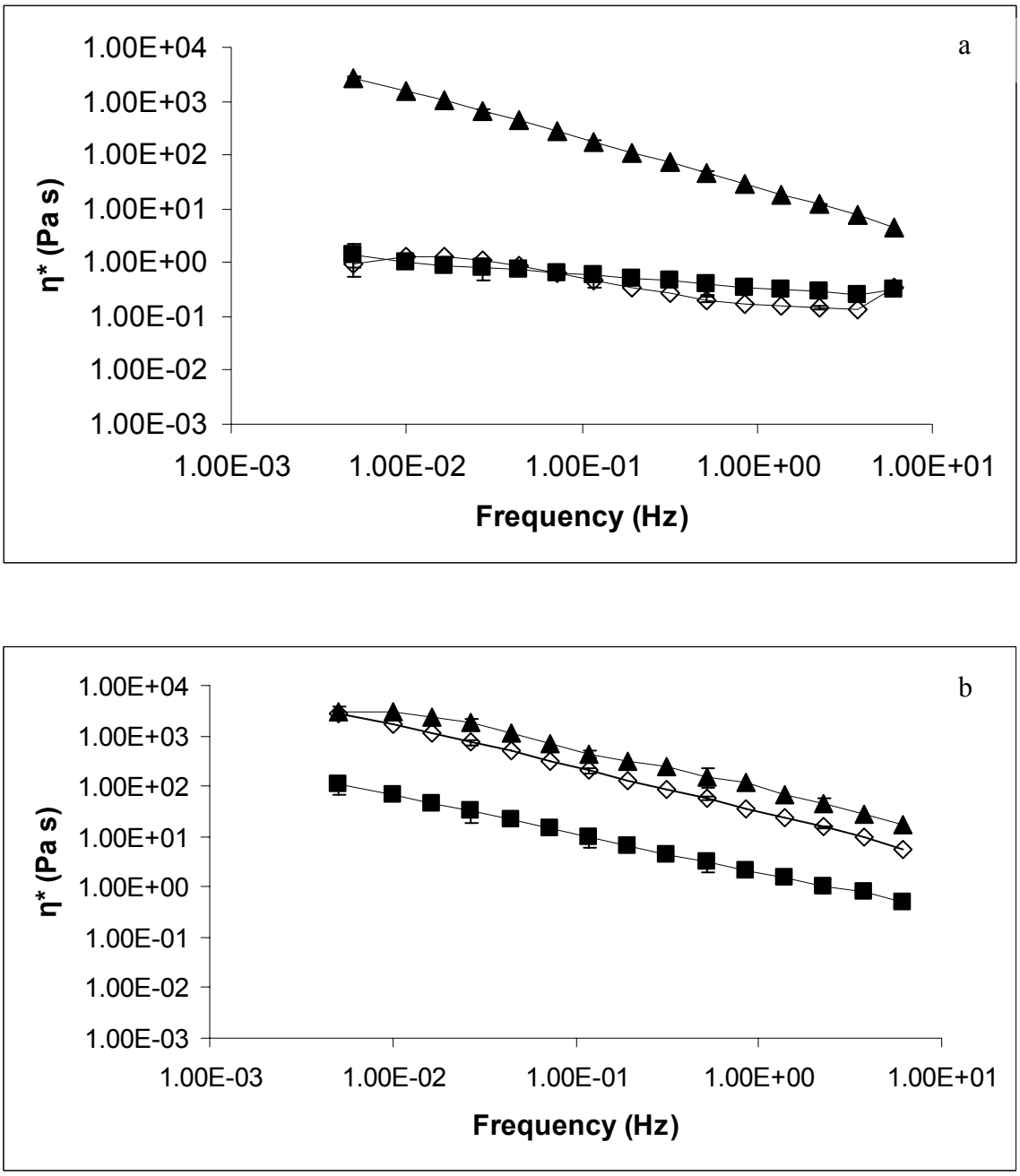


Figure 2.3. Complex viscosity (η*) as a function of frequency of Native SPI, 3% SPI, and 8% SPI to determine concentration effects.

Native SPI (◊), 3% SPI (■), and 8% SPI (▲) rehydrated at 8% protein (w/w) a. at 25°C. b. at 25°C after heating to 90°C. Error bars represent one standard deviation of three observations.

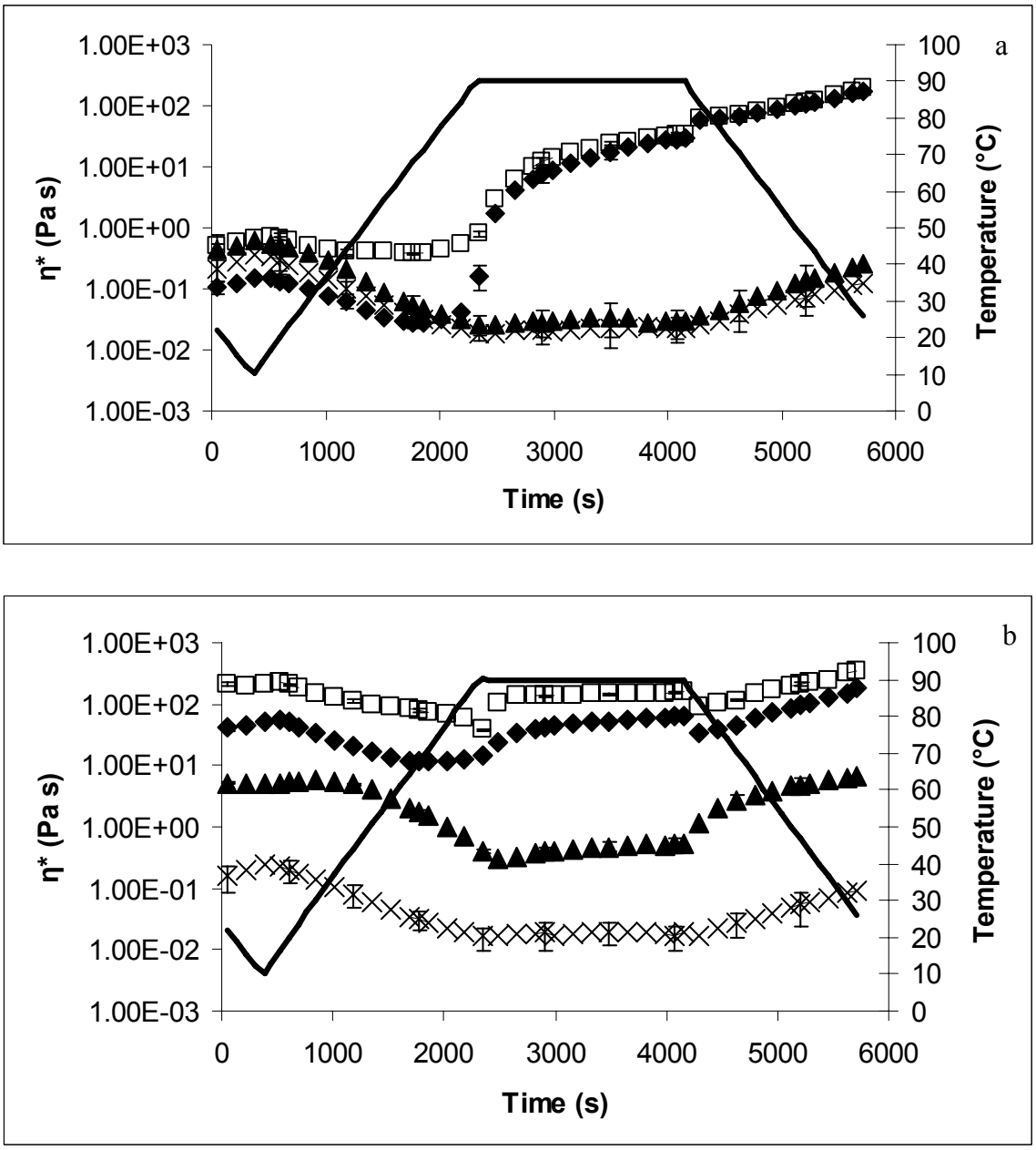


Figure 2.4. Temperature effects on the complex viscosity (η*) of a. native SPI rehydrated at 8% protein w/w with various denaturants. b. 8% SPI rehydrated at 8% protein w/w with various denaturants.

Native SPI and 8% SPI in water (□),w/ urea (▲), w/ DTT (♦), and w/ urea+DTT (x). Temperature changes are also recorded (—). Error bars represent one standard deviation of three observations.

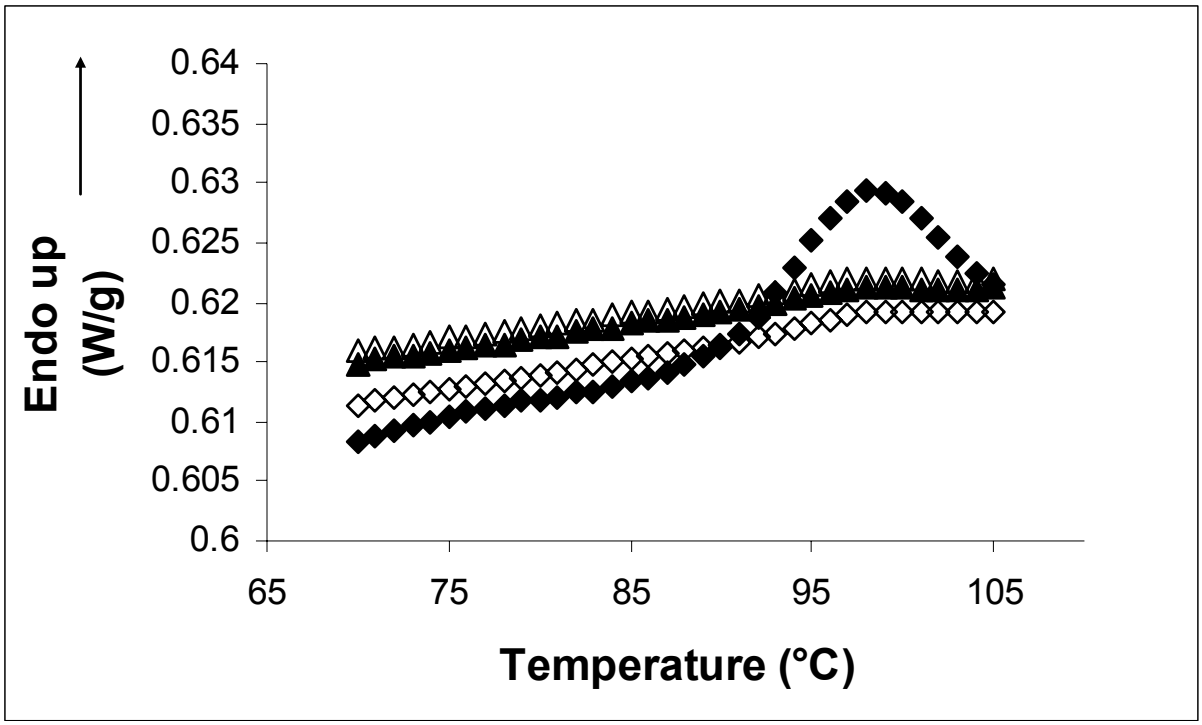
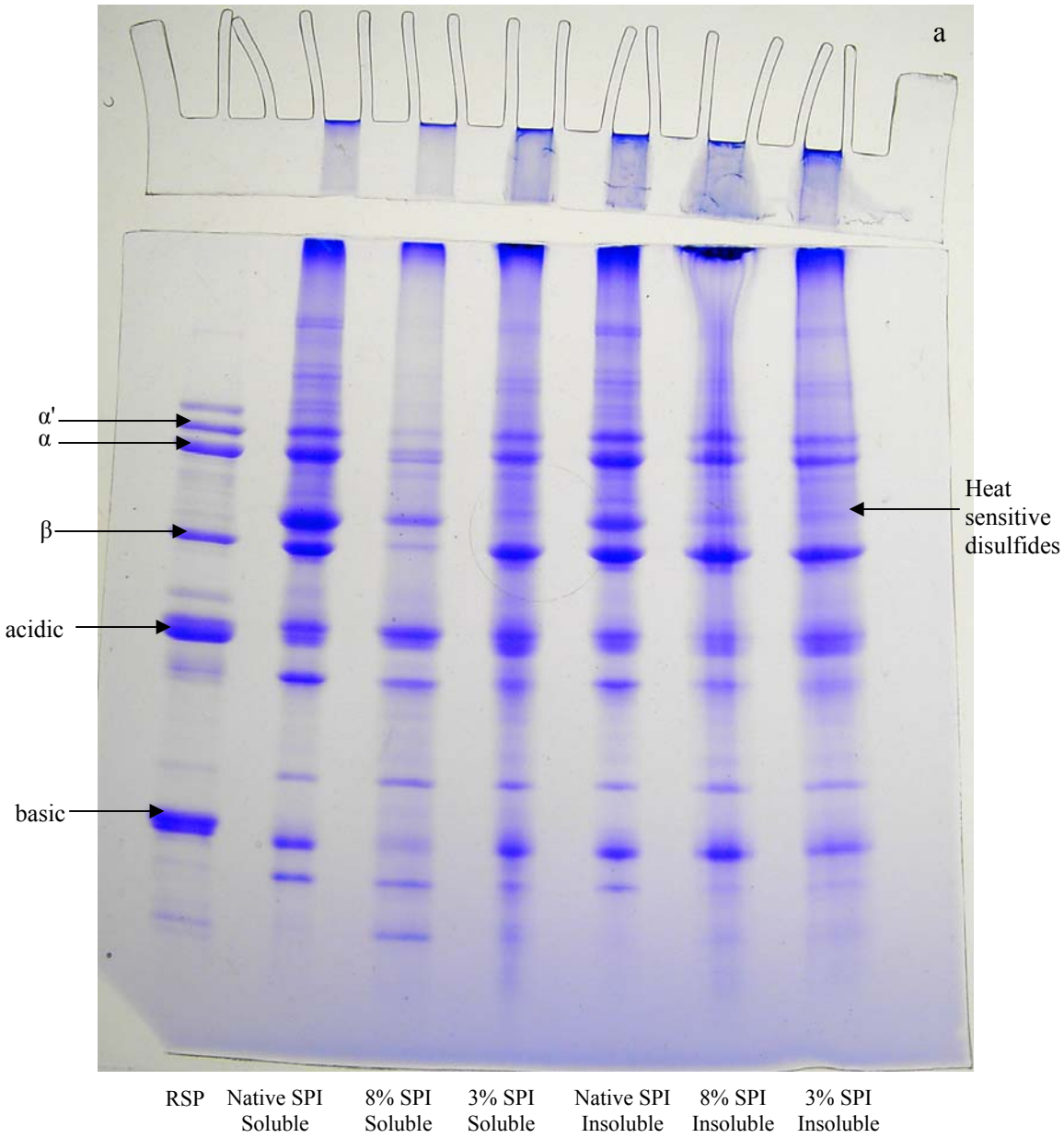


Figure 2.5. DSC thermogram showing heating curves for native and 3% heated SPI.

Each sample shows a first and second heating curve. All but Native 1 are fully denatured. Native 1 (♦), 3% heated 1 (▲), Native 2 (◊), 3% heated 2 (∆).

Figure 2.6. Iodoacetamide treated soy protein isolate a. after heating at 95°C for 3 hours and b. unheated control. Phase separation after two hours.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Heat
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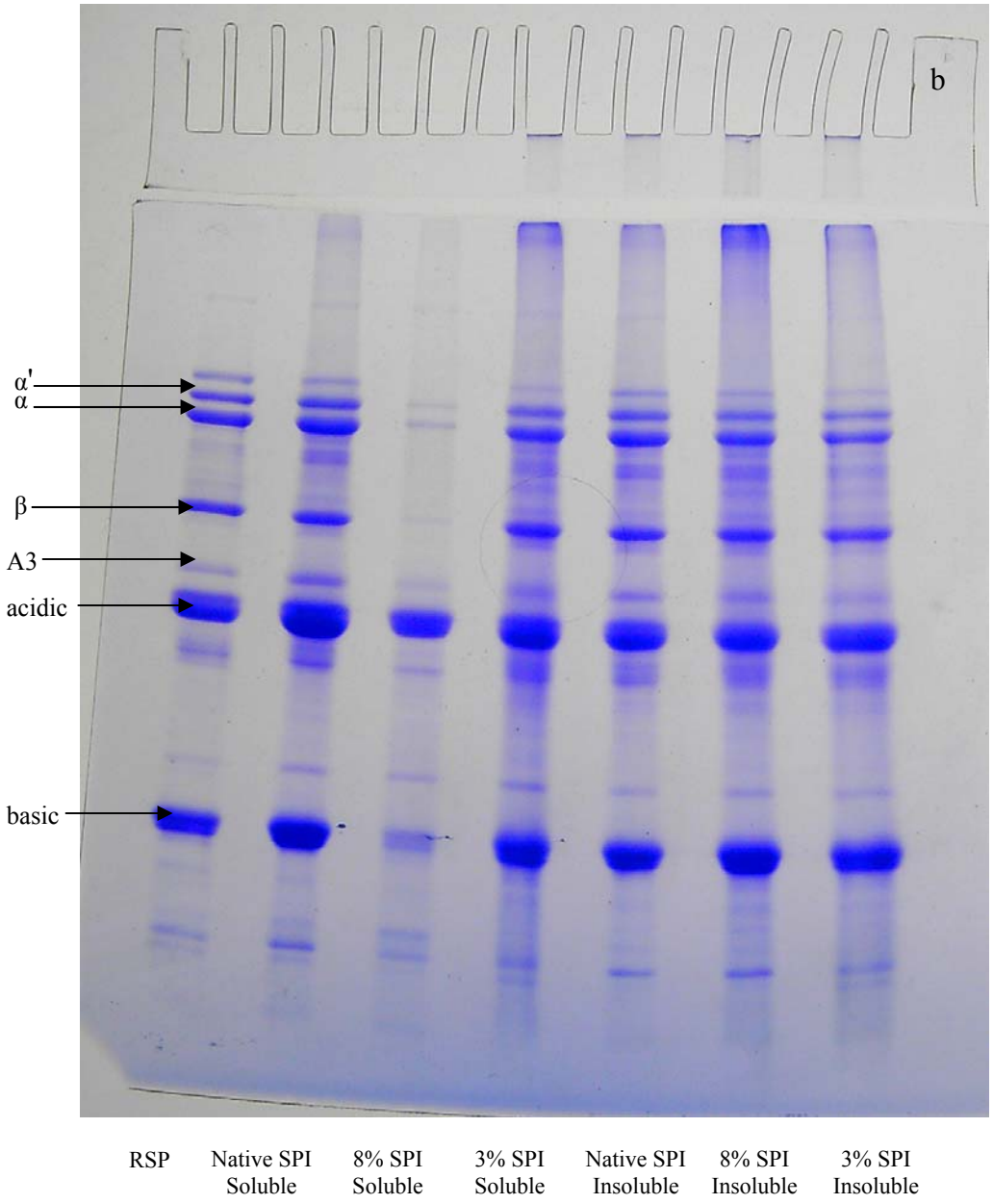

Figure 2.7. a. SDS-PAGE of soluble and insoluble fractions of Native, 8%, and 3% SPI without β-SH. b. SDS-PAGE of soluble and insoluble fractions of Native, 8%, and 3% SPI with β-SH, soluble and insoluble fractions separate. About 100 µg protein was loaded into each well.

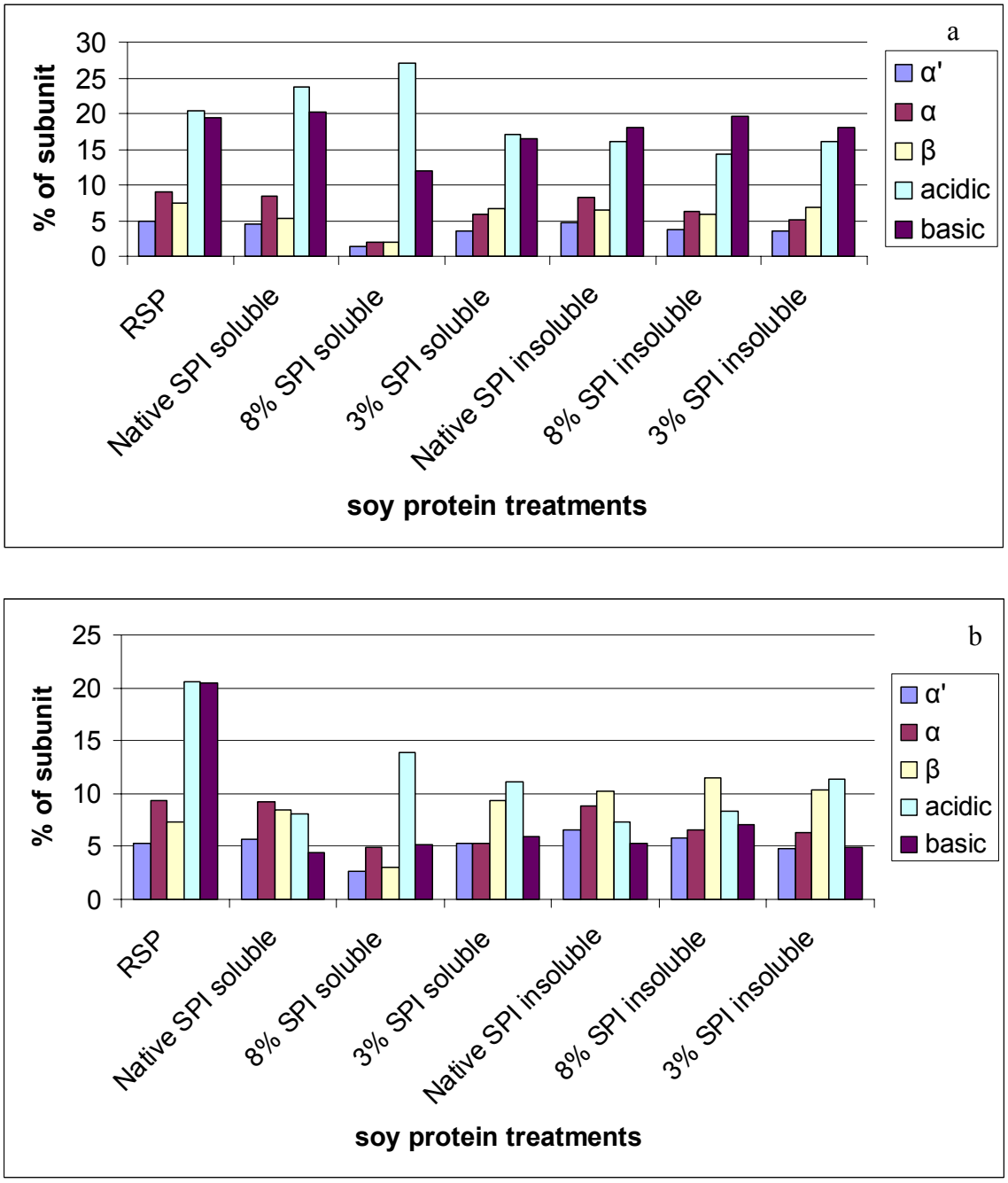

Figure 2.8. SDS-PAGE densitometer analysis showing percentage of each SPI subunit present in soluble and insoluble native, 8% SPI, and 3% SPI a. with β-mercaptoethanol b. without β-mercaptoethanol. About 100 µg of protein was loaded into each well.

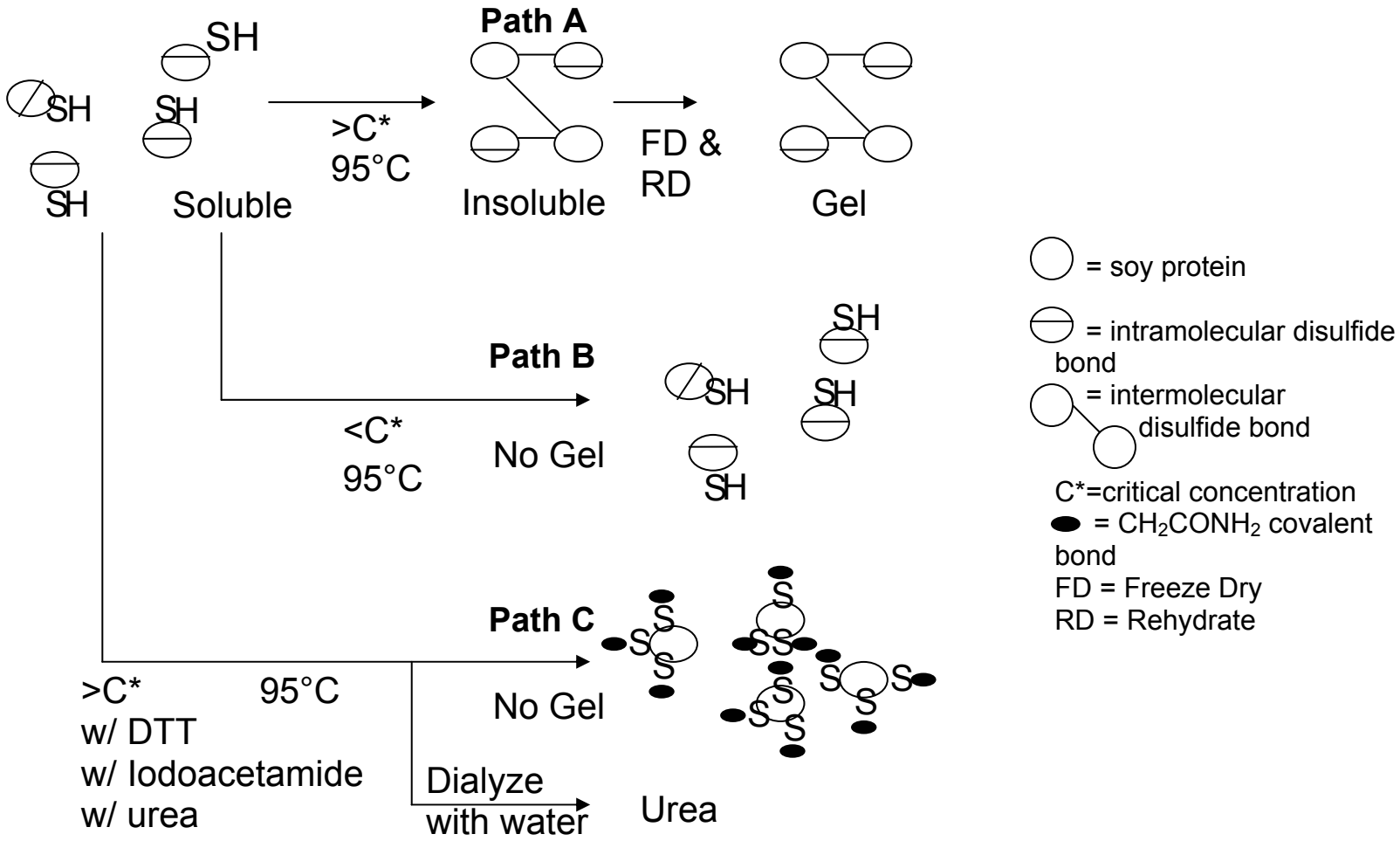

Figure 2.9. Proposed diagrammatic of disulfide interchange during heat induced gelation and effect of denaturants.

PART 3: The formation and functionality of soy protein-dextran and soy protein-mannose conjugates

3.1 Abstract

Soy protein isolate (SPI) was conjugated to mannose and two dextrans of different molecular weight (35-45 and 100-200 kDa) via the Maillard reaction to determine benefits from conjugation on viscosity and emulsifying properties. A soy protein isolate previously heat denatured and gelled at 8% protein (w/w) was also conjugated to dextran (100-200 kDa) via the Maillard reaction to determine the effect of prior denaturation on potential conjugation benefits. The Maillard reaction occurred using a dry heating procedure (2 hours, 100°C) and SPI: carbohydrate sample prepared at a 2:1 ratio. Complex formation between SPI and mannose was confirmed by examining protein and glycoprotein banding patterns, color changes, and a notable decline in pH from 6.8 to 6.3. In a similar manner, conjugation of SPI with dextran was evidenced in SDS-PAGE electropherograms by visualization of a broad smearing pattern, especially prominent in polyacrylamide gels stained for carbohydrate. This pattern suggested the formation of numerous sized glycoprotein products. The viscosity of the SPI: dextran glycoproteins was increased by approximately one order of magnitude over that measured for non-conjugated materials at 8% protein (w/v). However, the viscosity of previously denatured soy protein conjugate decreased an order of magnitude below the non-conjugated materials. Furthermore, SPI complexed with the 35-45 kDa dextran and the 100-200 kDa dextran produced emulsions with 31% and 81% less phase separation than non-conjugated samples. Complex formation between SPI and 100-200 kDa dextran also increased emulsion viscosity compared to SPI alone and non-conjugated SPI: dextran by nearly an order of magnitude at low shear rates. In contrast, SPI: mannose conjugates exhibited lowered viscosity and decreased emulsifying properties compared to SPI alone. This research demonstrates the potential of carbohydrate selection to achieve

desired performance characteristics of SPI conjugates to expand the utility of soy in food processing applications.

3.2 Introduction

Soy protein isolate (SPI) is a nutritious protein resource with functional properties that define its utility to the food industry. Soy protein isolate may be conjugated to reducing sugars via the Maillard reaction, with increasing molecular weight conjugates demonstrating increased ability to stabilize emulsions. Diftis et al. (2004) demonstrated that SPI conjugated in a 1:1 ratio with dextran (144 kDa) by dry heating at 60°C for one week improved the emulsion stability over nonconjugated SPI. The emulsions remained stabilized by the SPI-dextran conjugate in the presence of bovine serum albumin (BSA) or sodium dodecyl sulfate (SDS), but not in the presence of glycerol monostearate (GMS). Destabilization in the presence of GMS was attributed to protein interactions and floc formation. Diftis et al. (2005) further investigated this improvement in emulsion stability by comparing dry heating times for SPI using the same molecular weight (MW) dextran held at 60°C for one and three weeks. One week of heating produced conjugates that stabilized emulsions, but after three weeks of heating flocculation resulted that was attributed to bridging of the protein between oil droplets.

Dickinson and Semenova (1992) reported an increase in the emulsifying properties of 11S globulin that was conjugated to variable amounts of dextran (40 kDa and 500 kDa) with a dry heating procedure at 60°C for three weeks. However, heating for five weeks led to precipitation of the conjugate and loss of functionality. The five week heat treatment led to loss of solubility, while conjugation with dextran improved protein solubility and decreased the coarseness of the emulsion. The two MW dextrans demonstrated similar stability to

creaming when conjugated with 11S globulin. However, in non-conjugated 11S globulin-dextran emulsions, the higher MW dextran decreased the emulsion stability more than the lower MW dextran. The decrease in stability was likely due to more depletion flocculation associated with the larger MW dextran free in the solution.

Emulsifying stability (ES) and emulsifying capacity (EC) are two parameters that can be used to define emulsifying capabilities (Pearce and Kinsella, 1978). The ES and EC of an emulsion may not be directly related or predictive of each other (Webb et al., 1970). Emulsifying stability represents the measure of oil separation from an emulsion over a standard time period (Pearce and Kinsella, 1978). A creaming index may be used to measure ES by relating the height of the emulsified layer to the height of the original emulsion at predetermined time intervals (Keowmaneechai and McClements, 2002). Emulsifying capacity is a measure of the amount of oil that a standard amount of protein can emulsify under specified conditions (Pearce and Kinsella, 1978). Electrical conductivity was previously used to determine the EC of muscle proteins, whereby a sudden spike in the amperage represented emulsion destabilization (Webb et al., 1970). Previously the electrical conductivity method was used to measure the EC of whey protein dispersions (Firebaugh, 2004).

Based on previous findings that longer dry heating times (three or more weeks) at 60°C resulted in precipitation and loss of soy conjugate functionality (Diftis et al., 2005; Dickinson and Semenova, 1992), thermal treatments for two hours at a higher temperature (100°C) was hypothesized to produce conjugates with improved emulsifying properties over non-conjugated soy protein isolates. Higher temperatures reportedly increase the rate of the Maillard reaction (Stamp and Labuza, 1983; Martins and Van Boekel, 2005), leading to

faster production of conjugates and shorter heating times. Therefore, these methods would be more easily implemented in a time restricted commercial manufacturing scheme. Although 100°C is above the denaturation temperature of soy proteins, and denaturation is typically associated with a loss of solubility, no direct relationship between loss of solubility and loss of emulsifying properties has been established (Voutsinas et al., 1983). In fact, the ES of soy protein isolate decreased while the EC increased upon heating to 100°C or 121°C (Voutsinas et al., 1983).

For this work, mannose and two dextran reagents were used for conjugation with soy protein isolate via the Maillard reaction using dry heat conditions at 100°C for two hours in order to determine the effect of carbohydrate selection on defined aspects of ingredient functionality. The SPI was conjugated both in the native state and as a thermally denatured isolate which demonstrated gelation at ambient temperatures. These experiments were designed to determine the effect of previous denaturation and gelation on conjugation and resulting ingredient functionality.

3.3 Materials and Methods

3.3.1 Chemicals

Tris-Tricine sodium dodecyl sulfate (SDS) sample buffer, Tricine SDS running buffer, and Novex Colloidal Blue Staining Kit were purchased from Invitrogen Inc. (Carlsbad, CA). β-mercaptoethanol (β-ME) was purchased from Sigma Chemical (St. Louis, MO). Methanol was purchased from VWR, International (West Chester, PA). All other chemicals were of reagent or analytical grade. Corn oil was commercial grade. Distilled water or purified Milli-Q water (Millipore Corp., Billerica, MA) of 18 mΩ-cm resistivity was used for the preparation of all analytical reagents and buffers.

3.3.2 Extraction of Soy Protein Isolate

Soy protein isolate (SPI) was prepared from soybeans (*Glycine max L. Merr., cv.* Brim). The soybean seeds were ground in a Retsch centrifugal grinder, Model ZM100 (Newtown, PA), equipped with a 24-tooth rotor and a 1.0-mm stainless steel ring set at a motor speed of 14,000 rpm. This setting produced ground samples with a uniform particle size of less than 0.5 mm for efficient oil and protein extraction. Oil was extracted from the ground meal using a Soxhlet apparatus with warm hexane. Native protein was extracted batchwise from the defatted meal using a commercial procedure described by Lusas and Rhee (1995) and brought to pH 6.8 with NaOH. The final protein concentration was approximately 3% (w/v). The protein solution was lyophilized and then modified as described herein. The lyophilized SPI was ground in the centrifugal grinder with a 0.5mm sieve size, to produce SPI in a powdered form. The protein content of the lyophilized SPI was determined by the Dumas combustion method (Nielsen, 1998). Unless otherwise stated, all experiments and procedures were performed at room temperature.

3.3.3 Protein Modification

The SPI was separated into eight fractions (figure 3.1). The control SPI was rehydrated (8% solids w/v), followed by lyophilization and dry heating in an oven at 100°C for 2 hours (Treatment 1). The SPI: carbohydrate mixtures were prepared at a 2:1 ratio with either mannose, dextran (35-45 kDa), or dextran (100-200 kDa) and rehydrated (8% solids w/v), producing three distinct protein: carbohydrate dispersions. The SPI: mannose (Treatment 2) and the SPI: dextran (35-45 kDa) (Treatment 3) mixtures were lyophilized, and dry heated in an oven at 100°C for two hours. The SPI: dextran 100-200 kDa mixture was lyophilized and half of the powder was dry heated in an oven at 100°C for 2 hours

(Treatment 4) while the second half of the mixture was not heated (Treatment 5). The unheated material served as a control for determining the effect of conjugation. The denatured sample was prepared by rehydrating SPI (8% protein w/w) and wet heating at 95°C for 3 hours, followed by lyophilization. The wet heated SPI was separated into three portions, one with no further treatment (Treatment 6), one dry heated in an oven at 100°C for two hours (Treatment 7), and a third one mixed at a 2:1 ratio with dextran (100-200 kDa), rehydrated to 8% solids (w/v), lyophilized and dry heated in an oven at 100°C for two hours (Treatment 8).

3.3.4 Color Analysis

The color of all powders was determined based on the Lab color scale using a HunterLab DP-9000 with a D25 L optical sensor (Reston, VA).

3.3.5 SDS-Polyacrylamide Gel Electrophoresis

Protein concentrations were determined according to the Bradford method (Bradford, 1976) in order to load approximately the same amount of protein for each sample. The protein and glycoprotein profiles of the SPI and SPI conjugates were evaluated using an XCell SureLock Mini-Cell (Invitrogen, Inc., Carlsbad, CA). The samples (~25 mg/ml) were diluted 1:9 with 8% SDS, 0.9M Tris-Tricine sample buffer (Invitrogen Inc., Carlsbad, CA), to which 5% β-ME previously was added. The proteins were then dissociated at 100°C for 10 minutes. Each sample and a Multimark molecular weight marker (Invitrogen, Carlsbad, CA) was loaded into individual wells (about 2.5 µl protein per well) on a 10-20% Tris-Tricine gradient polyacrylamide gel (Invitrogen, Carlsbad, CA). Electrophoresis was accomplished under a constant current of 90mA per gel until the tracking dye reached the bottom of the gel.

Following electrophoresis, the samples were fixed and stained with a colloidal Coomassie Blue staining reagent (Invitrogen Inc., Carlsbad, CA) with agitation. The SPI conjugates were evaluated for carbohydrate attachment using a GelCode Glycoprotein Staining Kit (Pierce, Rockford, IL). Gels were dried according to the Novex MiniGel Drying Method (Invitrogen Inc., Carlsbad, CA).

3.3.6 Sample Preparation for Rheological Test

The eight treatments (figure 3.1) were all rehydrated (8% protein w/v) overnight at 4°C and tested within 48 hours of sample preparation.

3.3.7 Emulsion Preparation for Stability and Rheological Tests

The control SPI, SPI: dextran (35-45 kDa), SPI: dextran (100-200 kDa), and unheated SPI: dextran (100-200 kDa) samples were prepared according to a modified method by Firebaugh (2004). The powders were rehydrated at 5 mg/ml protein overnight at 4°C. Each solution was blended in a commercial Waring blender (Model 7010HG, Waring Products, Inc., New Hartford, CT) on high speed (22,000 RPM with no load) for 30 seconds at a 1:1 ratio with corn oil, resulting in a final protein concentration of 2.5 mg/ml. The blended mixtures were then homogenized in a GEA Niro Soavi S.p.A. Panda 2k bench top homogenizer (Parma, Italy) at 200 bar pressure for two passes. Each emulsion was prepared in triplicate.

3.3.8 Measurement of Viscosity

A Rheologica Stresstech Rheometer, ATS Rheosystems (Bordentown, NJ), was used to measure fluid properties of all test solutions and emulsions with a shear rate sweep (0.10 – 100 1/s). A smooth cup and bob was utilized and data were recorded for each sample in

triplicate. Glycoprotein samples were held at 25°C during testing, while emulsion samples were held at room temperature (22°C).

3.3.9 Particle Size Measurement

The particle size of the emulsions was determined with a Shimadzu SA-CP4 Centrifugal Particle Size Analyzer (Kyoto, Japan), in which case the measured density of the dispersed phase was 0.89 g/ml and the density of the continuous phase was 1.0 g/ml. The viscosity of the continuous phase was 1.0 mPa s.

3.3.10 Emulsifying Stability

The emulsifying stability (ES) was evaluated in duplicate for each emulsion according to a modified method by Keowmaneechai and McClements (2002). Ten milliliters of each emulsion was placed into a test tube and stored at ambient temperature (22°C) for seven days. Sodium azide (NaN_3) (0.02% w/v) was mixed into each tube to prevent microbial growth. The emulsions separated into two layers, with a transparent layer on the bottom and an emulsion layer on top. The height of the transparent layer was measured in milliliters and the creaming index was reported as:

$$\text{Creaming Index} = 100 \times [\text{Serum Layer (ml)} / \text{Original Emulsion (ml)}] \qquad \text{Equation 1}$$

3.3.11 Emulsifying Capacity

The emulsifying capacity (EC) was determined according to a modified method by Webb et al. (1979). The control SPI, SPI: mannose, SPI: dextran (35-45 kDa), SPI: dextran (100-200 kDa), and unheated SPI: dextran (100-200 kDa) were rehydrated (0.1% solids w/v) overnight at 4°C.

Two electrodes were secured close to the blade of a Model 5011 commercial Waring blender (Waring Products, Inc., New Harford, CT) to insure contact with the blended solution (Figure 3.2) according to the method by Firebaugh (2004). The electrodes were connected to an Omega HHM26 multimeter (Omega Engineering, Stamford, CT), which measured resistance as emulsions formed and destabilized. Data were collected, and emulsion destabilization was determined as the point at which resistance spiked. Protein solutions (200ml) were blended at high speed and corn oil was added at 1 ml/s with a Model 7553-80 Peristaltic Pump (Cole-Parmer Instrument Company) through Tygon tubing (4.4 mm internal diameter) that was secured close to the blender blade to insure that the oil was incorporated into the emulsion. Each solution was prepared in triplicate for measurement. The average volume (ml) of oil that was emulsified prior to the spike in resistance was reported as the emulsifying capacity.

3.4 Results and Discussion

The L value from the Lab color scale was used to measure sample color changes indicative of melanoidins produced as a result of Maillard browning (Izydorczyk, 2005; Nursten, 2005). The L value indicates luminance on a scale from zero to 100 (dark to light). The SPI: mannose sample displayed extensive browning as evidenced by a visual color change from off-white to caramel brown, corresponding to a change in L value from 83.51 for the control SPI to 76.64 for the conjugate. Complex formation was accomplished at pH 6.8, experimental conditions that facilitate the kinetics of the reaction (Nursten, 2005). However, the pH of the SPI: mannose conjugate shifted from 6.8 to 6.3, characteristic of high reaction temperatures whereby acidic by-products of the Maillard reaction may form (Cabodevila, 1994). The SPI: dextran samples did not darken in color or shift in pH. Instead

the addition of white dextran powder (L value 100) increased the L value from 83.51 to 86.16 for the unheated SPI: dextran mixture while an average L value of approximately 89.00 was noted for the heated SPI: dextran conjugates. Since these samples were all prepared on an equal protein to carbohydrate weight basis, the SPI: mannose conjugate contained higher molar ratios of carbohydrate to protein compared to that of either SPI: dextran sample, likely resulting in higher amounts of glycoconjugate formed. Increased SPI: mannose conjugation would also be expected to cause visual color changes that often accompany the Maillard reaction, as were observed. Since no color changes were noted with the SPI: dextran samples, electrophoresis was performed to determine whether or not the Maillard reaction occurred as evaluated by SDS-PAGE.

3.4.1 Conjugation Verification with SDS-PAGE

Glycoprotein profiles observed after SDS-PAGE showed that SPI was conjugated to mannose, dextran (35-45 kDa), and dextran (100-200 kDa) via the Maillard reaction with a dry heating procedure (100°C, 2 hours). SPI: mannose conjugation was evident with respect to all major subunits and displayed an upward shift in the average molecular weight of each fraction (α', α, β, acidic, and basic) (figure 3.3a). This shift also corresponded to the one observed in glycoprotein stained gels, which exhibited a more notable acidic and basic band than in the control SPI (figure 3.3b).

The SPI: dextran (35-45 kDa) and (100-200 kDa) glycoprotein profiles revealed a broad smearing pattern at the entrance to the separating gel, supporting the formation of high molecular weight conjugates (figure 3.3a). The SPI: dextran (35-45 kDa) conjugates migrated further into the gel than the larger SPI: dextran (100-200 kDa) conjugates, which was to be expected based on the molecular size of the dextrans. This broad staining pattern

was not seen in unheated samples of SPI: dextran (100-200 kDa) (data not shown), suggesting the absence of complex formation. However, the denatured SPI: dextran (100-200 kDa) treatment also displayed a broad staining pattern, suggesting complex formation (data not shown). Similar dense staining patterns were previously observed with pronase-treated gluten complexed with dextran (Kato et al. 1991), ovalbumin complexed with dextran (Kato et al. 1990), and SPI complexed with dextran (Diftis and Kiosseoglou, 2004).

The glycoproteins evidenced with control SPI are due to natural glycoproteins formed between 7S subunits and mannose (Koshiyama, 1969). Many protein bands did not change their migration patterns, demonstrating that not all amine groups become involved in conjugation during the dry heating procedure.

Neither mannose, dextran (35-45 kDa), nor dextran (100-200 kDa) migrated into the resolving gel (figure 3.3 a and b), as would be expected since these carbohydrates were neutrally charged. Therefore, the dense staining patterns at the gel interface were attributed to glycoprotein formation.

3.4.2 Effect of Conjugation on Viscosity

The viscosity of the SPI: dextran (100-200 kDa) conjugates prepared at 8% protein (w/v) demonstrated the highest viscosity of all samples, exhibiting a viscosity that was nearly an order of magnitude greater than the unheated SPI:dextran (100-200 kDa) dispersion (figure 3.4). The unheated SPI: dextran (100-200 kDa) demonstrated a viscosity decrease compared to the control SPI. The SPI: dextran (35-45 kDa) conjugates demonstrated an average viscosity similar to that of the control SPI, while the SPI: mannose conjugates showed a lower shear plateau that was nearly two orders of magnitude below that of the

control SPI at low shear rates. All samples exhibited shear thinning behavior with increasing shear rates.

The SPI: dextran (100-200 kDa) conjugates formed the most viscous dispersions, despite the smaller number of dextran molecules that were present for conjugation, based on a molar concentration. These results suggested that an increase in viscosity of these glycoprotein dispersions was dependant on the formation of high molecular weight complexes and was enhanced by the degree of branching (dextran).

The drop in viscosity seen with SPI: mannose conjugates was accompanied by sedimentation. Thus, the extent of conjugation under these experimental conditions may interfere with steric stabilization, resulting in sedimentation and decreased flow behavior. Previously, a high degree of conjugation was reported to produce insoluble aggregates (Kato, 2002).

The viscosity of the denatured SPI, gelled with a wet heating procedure at 8% protein (w/w), was similar to the control SPI and the SPI: dextran (35-45 kDa) conjugates. The denatured SPI gelled at ambient temperatures and exhibited immediate dispersibility in water. Therefore, conjugation to this type of SPI was hypothesized to further increase viscosity and gelation capabilities. However, dry heating of the denatured SPI fraction, in the presence and absence of dextran (100-200 kDa), lowered the viscosity by more than one order of magnitude. Also, the denatured SPI prepared with and without dextran 100-200 kDa resulted in sedimentation after dry heating, a phenomenon which requires further investigation.

The steady shear tests used to test the glycoprotein solutions break up any network formed and determine the viscosity of the sample, as opposed to oscillatory tests, which

maintain the network and test its solid and viscous properties. While steady shear viscosity (η) obtained from steady shear tests and apparent viscosity (η*) obtained from oscillatory tests may be related according to the Cox-Merz rule (Equation 2), which states that η and η* are almost equal when shear rate and frequency are equal (Steffe, 1996), the η parameter is of interest to this work.

$$\eta^* = \eta|_{\omega=\dot{\gamma}} \qquad \text{Equation 2}$$

Higher viscosity in the continuous phase of an emulsion may increase stability based on Stokes Law (Equation 3), where v is the velocity of the droplets in the continuous phase, r is the radius of the droplet, Δρ is the change in density between the oil and continuous phase, g is gravity, and η is the viscosity of the continuous phase.

$$v = 2r^2(\Delta\rho)g/9\eta \qquad \text{Equation 3}$$

Stoke's Law indicates that a higher continuous phase viscosity will decrease the velocity or movement of the dispersed oil droplets, therefore encounters between the droplets will decrease over a standard period of time and the emulsions will remain stable longer. Since a decrease in viscosity was observed with denatured SPI and denatured SPI: dextran conjugates after dry heating, further experimentation with emulsions was only performed with native proteins.

3.4.3 Effect of Conjugation on Emulsifying Stability (ES)

The particle size of freshly prepared emulsions stabilized with control SPI, SPI: dextran (35-45 kDa), SPI: dextran (100-200 kDa), and unheated SPI: dextran (100-200 kDa) was similar to each other over three replications (Table 3.1). Therefore, the creaming index may be attributed to the stabilizing ability of the emulsifier itself rather than differences in

particle size per se. On average, the SPI: dextran (35-45 kDa) emulsions produced a 31% lower creaming index than the control SPI emulsions. The SPI: dextran (100-200 kDa) stabilized emulsions produced an 81% lower creaming index than the control SPI emulsions. The unheated SPI: dextran (100-200 kDa) emulsions produced a similar creaming index to the control SPI emulsions. However, based on the SDS-PAGE results, conjugation was not achieved in the unheated SPI: dextran (100-200 kDa) samples (data not shown). Thus, depletion flocculation may occur with the unheated SPI: dextran (100-200 kDa) sample caused by free dextran molecules, as previously described by Dickinson and Semenova (1992). In the SPI: dextran (100-200 kDa) conjugates, the larger molecular weight dextran likely produced a thicker steric layer around the oil droplets due to a larger extent of branching, resulting in improved functionality of these conjugates with respect to emulsifying stability. Steric stabilization of high molecular weight protein–carbohydrate conjugates was previously associated with emulsion stabilization (Dickinson and Semenova, 1992; Kato, 2002; Diftis et al., 2005).

The SPI: mannose conjugates did not stabilize the pre-emulsion long enough to effectively homogenize the sample. Therefore, the SPI: mannose conjugates did not demonstrate ES potential, and were excluded from further experimentation. The inability of SPI: mannose conjugates to stabilize an emulsion may be due to the low molecular weight and lack of branching associated with mannose sugars. These characteristics may limit steric effects that typically occur with larger conjugates. Another possibility for destabilization may be depletion flocculation caused by a large number of free mannose molecules present in the continuous phase. A larger number of these free sugar moieties would be expected in SPI: mannose solutions as compared to SPI: dextran solutions, which were prepared at a

lower molar sugar concentration. An ideal ratio that minimizes the amount of free polysaccharide and prevents depletion flocculation, while providing optimal conjugation and steric stabilization, has been found to be different for each protein-polysaccharide combination (Dickinson and Semenova, 1992).

The inability of SPI: mannose conjugates to stabilize an emulsion may be correlated with the decreased viscosity of the dispersions. As previously stated, the molar concentration of mannose was higher than that of dextran, correlating with increased conjugation potential. Similarly a high degree of protein: carbohydrate complex formation may interfere with the emulsifying effect of the protein, leading to aggregation and insolubility (Dickinson and Semenova, 1992; Kato, 2002). Based on Stoke's Law (Equation 3), a higher viscosity in the continuous phase, such as that seen with the SPI: dextran (100-200 kDa) conjugates will decrease the velocity of the oil droplets, leading to an increase in emulsion stability. Therefore, the branched dextran conjugates may stabilize emulsions simply by increasing the viscosity of the dispersed phase, which the SPI: mannose conjugates were not able to do.

The viscosity of the emulsions was inversely proportional to the ES behavior, with the highest viscosity emulsions corresponding to the lowest creaming index. On average, the viscosity of the SPI: dextran (100-200 kDa) emulsion was nearly an order of magnitude higher than the control SPI at low shear rates, likely due to the high degree of branching associated with the molecular structure of dextran (figure 3.5). The SPI: dextran (35-45 kDa) emulsions exhibited an intermediate viscosity between that of SPI: dextran (100-200 kDa) conjugate emulsions and the control SPI emulsions, while the average viscosity of the unheated SPI: dextran (100-200 kDa) emulsions was lower than the control SPI emulsions. All emulsions displayed shear thinning behavior that was very similar to the 8% protein

(w/v) conjugate dispersions. Shear thinning was also observed in emulsions stabilized by SPI: dextran conjugates that were formed through dry heating at 60°C for zero, one, or three weeks (Diftis et al., 2005). Diftis et al. (2005) also found that the unheated SPI: dextran mixture produced a more solid-like emulsion network due to depletion flocculation, while a mixture heated for one week produced a liquid-like, sterically-stabilized emulsion, and a third mixture heated for three weeks produced a solid-like emulsion attributed to bridging flocculation. The more solid-like emulsions were found to be more stable in the long run, which supported current findings presented here, in which the higher viscosity emulsions were correlated with greater ES.

3.4.4 Effect of Conjugation on Emulsifying Capacity (EC)

The SPI: dextran (100-200) kDa conjugate did not display a significant difference in EC compared to either the control SPI or the SPI: dextran (35-45 kDa) conjugates (figure 3.6). The EC of the SPI: mannose conjugates was approximately half that of the SPI: dextran (100-200 kDa) conjugates. The EC behavior for SPI: mannose was expected based on the lack of ES ability, which may be due to interference with the protein steric stabilization effects by the high degree of mannose conjugation, promoting protein-protein interactions and the coalescence of droplets.

3.5 Conclusion

Soy protein isolate was conjugated to mannose and to dextran of two molecular weights via the Maillard reaction at 100°C for two hours. Increasing the molecular weight of the carbohydrate reactant increased the viscosity of the resultant glycoprotein dispersions. Also, the viscosity was enhanced using native SPI starting materials over denatured SPI starting materials. The emulsion viscosity and ES of the conjugates prepared with native SPI

depended on the size of the carbohydrate reactant, with the small SPI: mannose conjugates providing no detectable emulsion stabilizing effect while the SPI: dextran (35-45 kDa) conjugate emulsions demonstrated an increase in stability and viscosity over the control. The SPI: dextran (100-200 kDa) conjugate emulsions exhibited the greatest stability and viscosity of all test samples. The SPI: dextran conjugates did not differ from the control SPI dispersions with respect to EC, but the SPI: mannose conjugates exhibited a lower EC, which was to be expected based on the lack of ES. These results suggested that higher molecular weight polymers were essential for providing improved emulsion stability and viscosity while heat denatured soy protein starting materials exhibited decreased viscosity and sediment formation.

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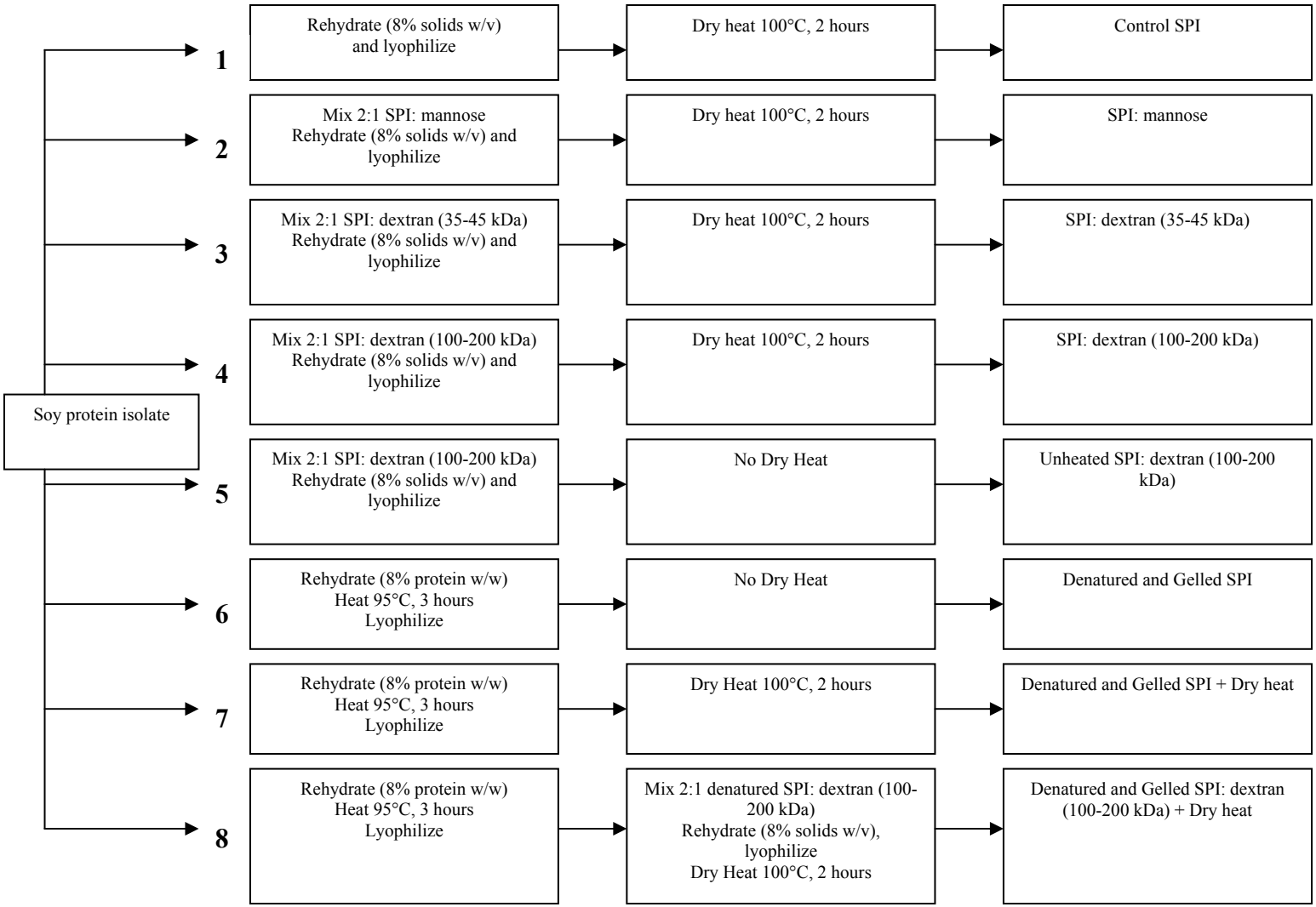

Figure 3.1. Sample preparation.

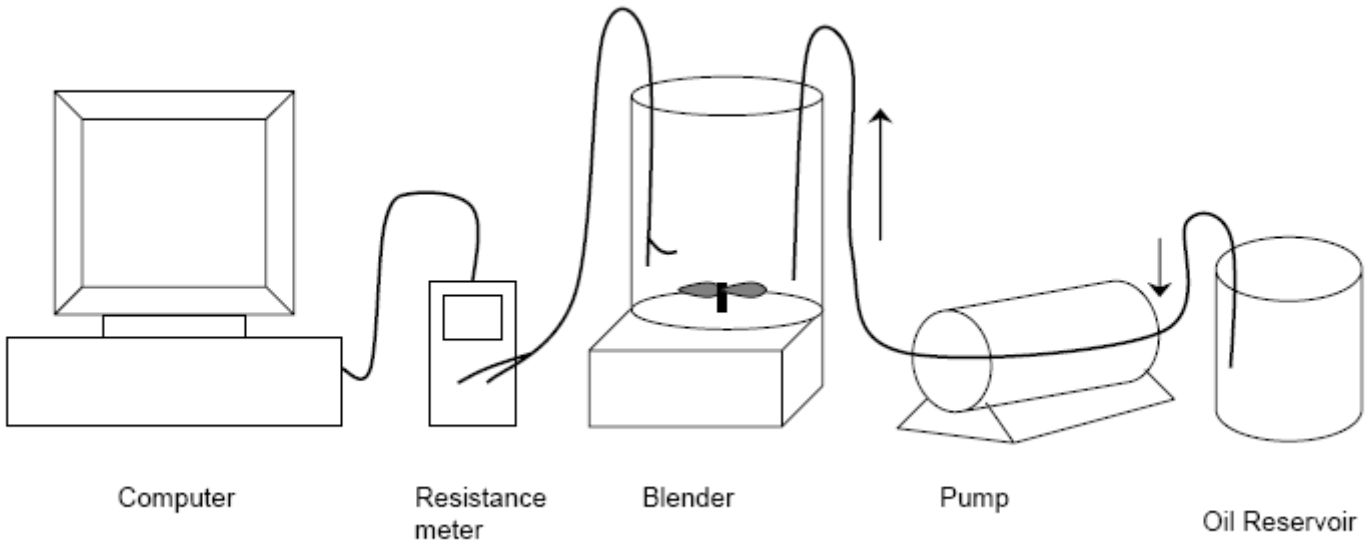

Figure 3.2. Schematic of emulsion forming and electrical conductivity measuring apparatus (Firebaugh 2002).

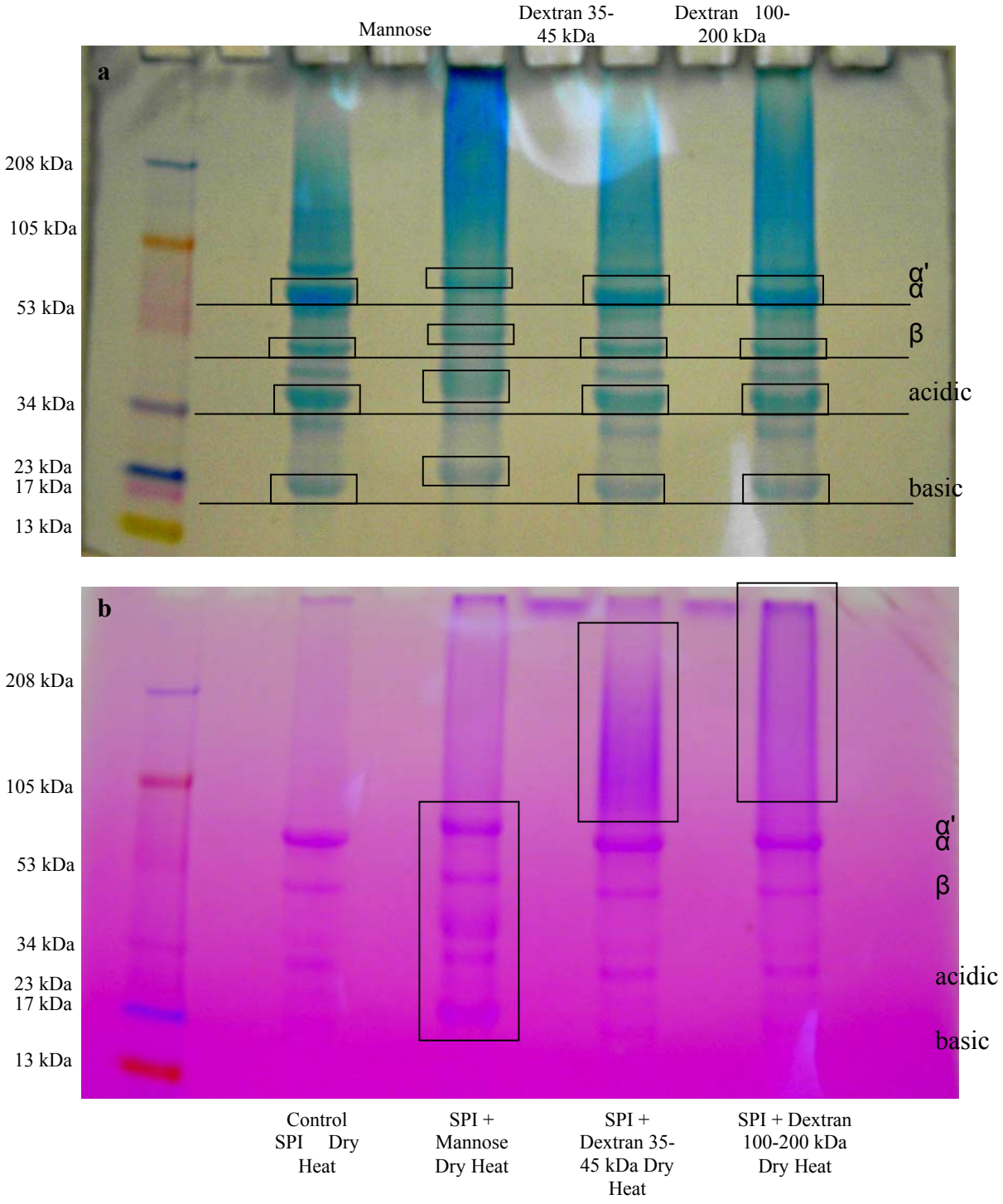

Figure 3.3. SDS-PAGE gel stained for a. protein bands and b. carbohydrate containing bands. Carbohydrate lanes are labeled at the top of both gels and protein: carbohydrate lanes are labeled at the bottom of both gels.

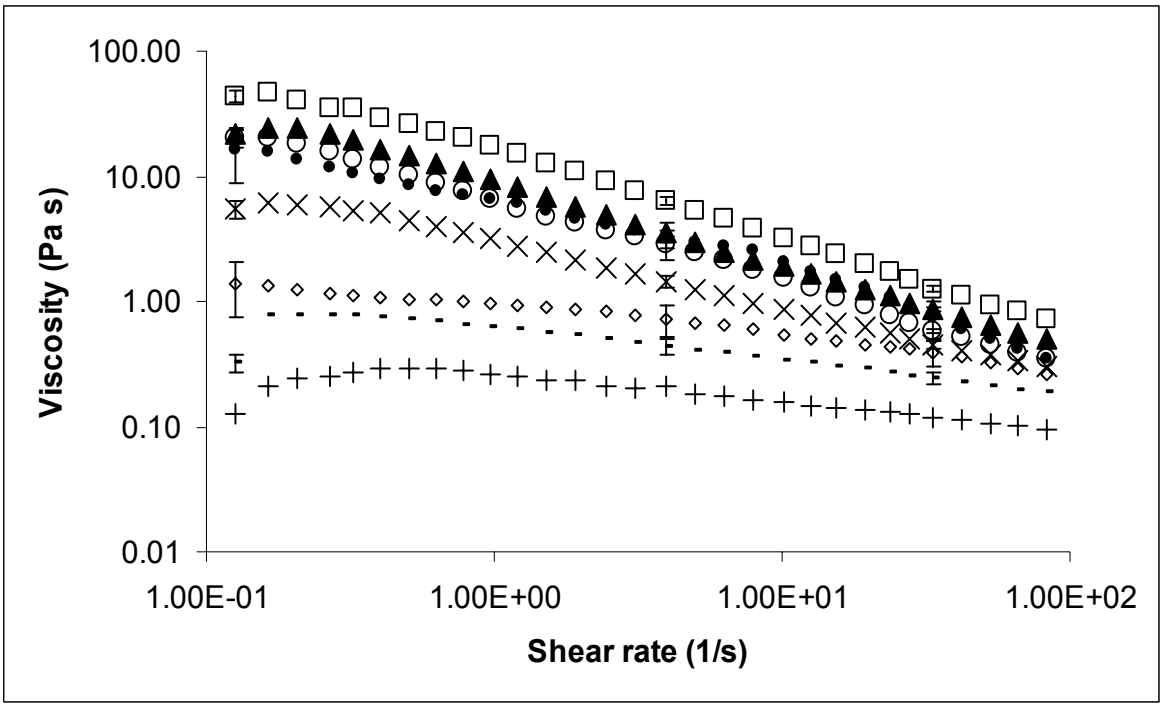

Figure 3.4. Viscosity of 8% soy protein (w/v) free or conjugated with several MW carbohydrates at a 2:1 ratio.

Control SPI (○) ; SPI: Mannose (+); SPI: Dextran 35-45 kDa (▲), SPI: Dextran 100-200 kDa (□); Unheated SPI: Dextran 100-200 kDa (x); denatured SPI (●); denatured SPI dry heat (◊); denatured SPI dry heat: Dextran 100-200 kDa (-). Error bars represent one standard deviation.

Table 3.1.

Stability of emulsions containing 0.25% soy protein, free or conjugated

Treatments	Creaming index ± one standard deviation	Particle Size (μm) ± one standard deviation
Control SPI	11.00 ± 4.36	8.75±0.83
SPI: Dextran 35-45 kDa	7.58 ± 1.38	8.29±1.36
SPI: Dextran 100-200 kDa	2.12 ± 0.89	10.45±0.96
SPI: Dextran 100-200 kDa No heat	12.67 ± 2.36	9.32±2.59

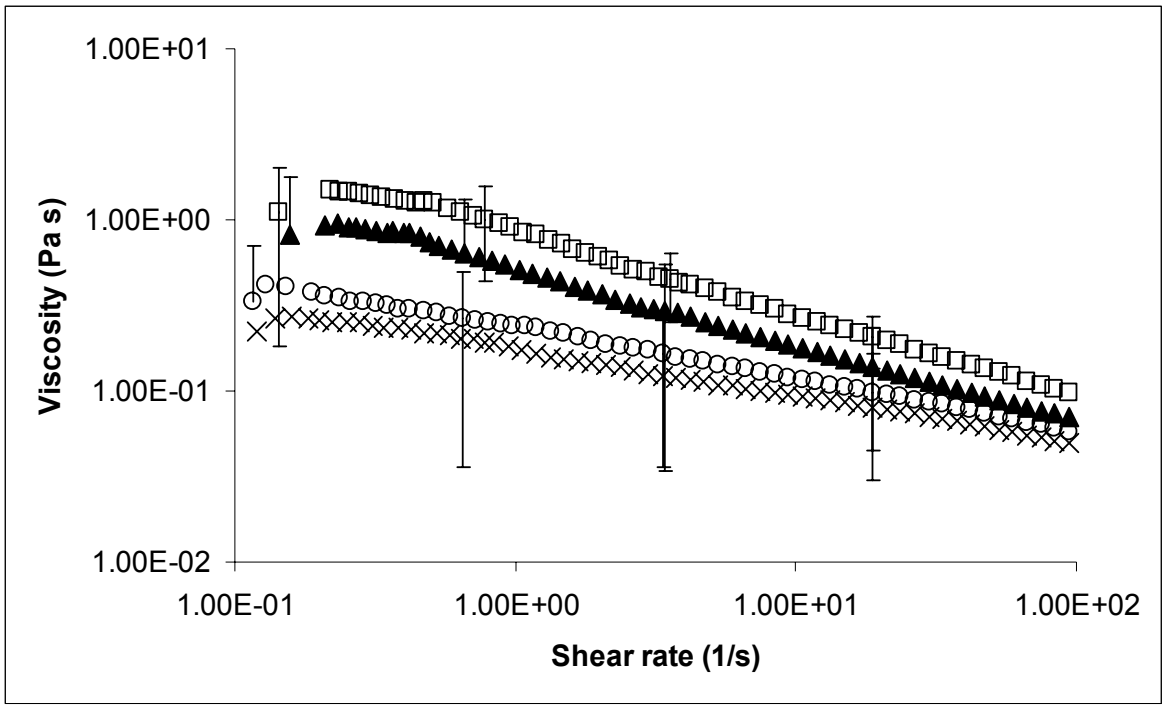

Figure 3.5. Viscosity of emulsions containing 0.25% soy protein, free or conjugated.

Control SPI (○); SPI: Dextran 35-45 kDa (▲); SPI: Dextran 100-200 kDa (□); Unheated SPI: Dextran 100-200 kDa (x). Error bars represent one standard deviation.

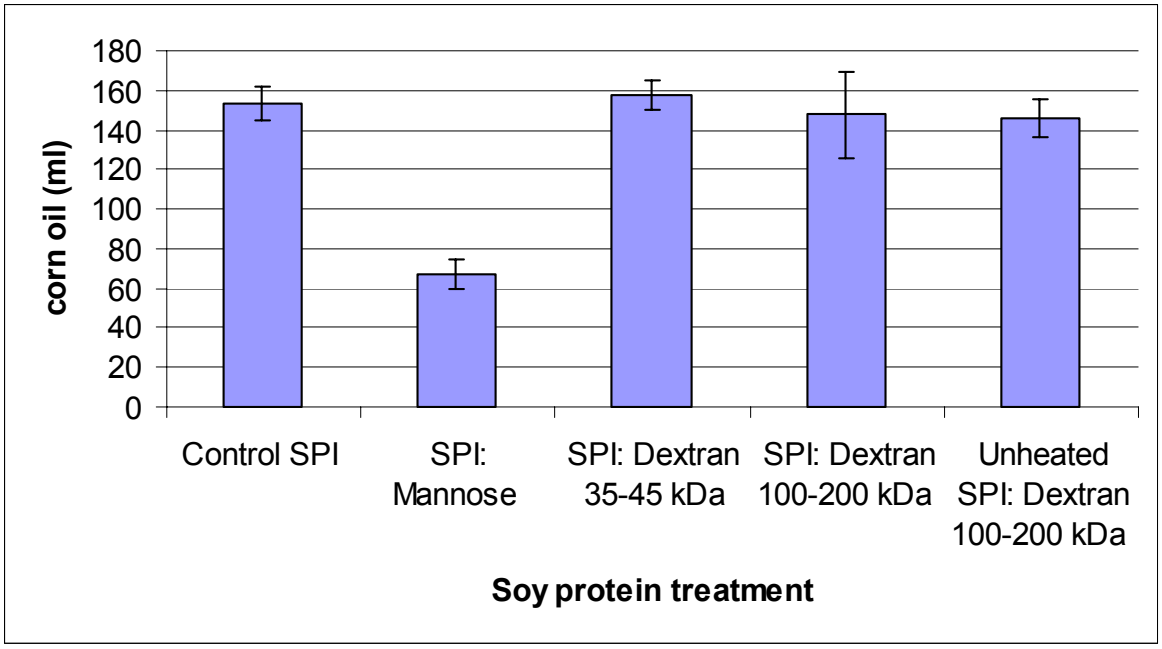

Figure 3.6. Emulsion capacity of solutions containing 0.1% soy protein (w/v), free and conjugated

APPENDICES

I. Acronyms

Soy Protein Isolate	SPI
Kilodaltons	kDa
sodium chloride	NaCl
sodium thiocyanate	NaSCN
β-mercaptoethanol	β-ME
dithiothreitol	DTT
N-ethylmaleimide	NEM
sodium borohydride	$NaBH_4$
sodium sulfite	Na_2SO_3

II. Demonstration of elasticity for SPI heated at 8% protein (w/w) for 3 hours at 95°C, shown here at 25°C.