ABSTRACT

MCKLEM, LACEY KAY. Investigation of molecular forces involved in gelation of commercially prepared soy protein isolates. (Under the direction of Dr. Tyre Lanier and Dr. Prachuab Kwanyuen)

Gelling behavior of soy protein isolates (SPI) commercially prepared from two soybean cultivars, *Prolina* and *Brim*, was studied. SPI (prepared at 12% protein w/w) were predenatured by commercial processing and gelled immediately upon hydration, prior to heat treatment. This research first investigated the effects of temperature, holding time, and reheating on thermal reversibility and reformability of these commercial SPI gels. Secondly, chemical reagents were added to determine the molecular forces contributing to their gelation.

Small strain rheology was used to determine the effect of temperature (40 – 90 ºC), holding time at elevated temperature (0 or 30 min), and reheating on thermal reversibility of *Prolina* and *Brim* gels. Final gel rigidity (measured by storage modulus G′) increased with increasing endpoint temperature, and holding at each endpoint temperature further increased the final gel rigidity. During heating, all gels exhibited a decrease in G′, which was largely reversible upon cooling. *Prolina* and *Brim* gels exhibited increases in G′ during holding at temperatures ranging from 50 – 90 ºC. Reheating did not enhance or diminish the gel rigidity.

Vane fracture testing was utilized to investigate temperature effects and reformability of cooked gels. Fracture stress of heated and cooled gels was significantly higher than initial gels, while fracture deformation was significantly lower. Cooked gels were rechopped and gels reformed at 10 ºC, again strengthening upon subsequent heating

and cooling. Again, additional thermal treatment did not enhance or diminish gel properties.

To investigate contribution of molecular forces, the chemical reagents urea (weakens hydrogen bonding and hydrophobic interactions) and dithiothreitol (DTT) (reduces disulfide bonds) were added to *Prolina* and *Brim* SPI and resulting gelling behavior was monitored during small strain rheology. Control (deionized water) and urea-added samples gelled initially and G′ decreased upon heating to 80 ºC, whereas DTT-added samples did not gel initially. Gel rigidity of both control and DTT-added samples increased during holding at 80 ºC. Final gel rigidity of urea- and DTT-added samples was lower than control samples; the sum of the final G′ values for the urea- and DTT-added samples neared the final G′ value of the control sample for both *Prolina* and *Brim* gels. Urea had a more detrimental effect on gel rigidity of *Brim* gels, whereas DTT had a similar effect on *Prolina* and *Brim*.

Prolina and *Brim* gels (unheated and heated) were solubilized in various chemical reagents. Addition of urea + DTT completely solubilized all samples, while solubilization in either reagent alone was greater than solubilization with the control treatment. More protein was solubilized from gels that exhibited lower gel rigidity.

Results from this investigation indicate that hydrogen bonding, hydrophobic interactions, and disulfide bonding all play roles in commercial SPI gelation. Disulfide bonding is important in the initial gel network as indicated by a high degree of solubilization in DTT and formation of gel prior to heating by pastes containing urea. Disulfide bonds are not essential for gel formation, but their presence adds rigidity to the gel network. Hydrogen bonding and hydrophobic interactions, however, are the primary

forces contributing to gelation of these commercially prepared SPI. This is evidenced by the extensive reformability of the gels as well as the similar effects of heating and holding on *Prolina* and *Brim* gels. More specifically, increases in G′ during holding and cooling were attributed to hydrophobic interactions and hydrogen bonding, respectively.

INVESTIGATION OF MOLECULAR FORCES INVOLVED IN GELATION OF COMMERCIALLY PREPARED SOY PROTEIN ISOLATES

by
LACEY KAY MCKLEM

A thesis submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the Degree of
Masters of Science

DEPARTMENT OF FOOD SCIENCE

RALEIGH

2002

APPROVED BY:

Dr. Jonathan C. Allen	Dr. Christopher R. Daubert
Dr. Tyre C. Lanier Co-chair of Advisory Committee	Dr. Prachuab Kwanyuen Co-chair of Advisory Committee

BIOGRAPHY

Lacey Kay McKlem was born on March 24, 1978 in Columbus, Nebraska to Leroy and Lois Johannes. Lacey attended Immanuel Lutheran School (K-8) and graduated from Columbus High School in 1996. She started college at the University of Nebraska – Lincoln in the fall of 1996, majoring in Food Science & Technology with a Science option. Lacey graduated in May 2000 with high distinction and completed an undergraduate thesis project for the University Honors Program. In August 2000, Lacey began her graduate program in Food Science at North Carolina State University under the direction of co-advisors Dr. Tyre Lanier and Dr. Prachuab Kwanyuen.

ACKNOWLEDGEMENTS

I would like to thank Dr. Lanier for his constant support and advice. His unending curiosity always pushed me to my intellectual limits and made this research project very rewarding. I would also like to thank Dr. Kwanyuen for his assistance and direction in the lab, as well as his ability to keep me motivated and entertained. I am grateful to Dr. Daubert for keeping an open door and addressing all of my rheological questions.

Special thanks to Penny Amato for all of her technical assistance and interest in this project and for her willingness to help me at any time. Also, I am grateful for her patience in listening to my problems and putting up with me on my "talkative" days. I would also like to thank my labmates – Alex, for always helping me out when I needed him and Patricio, for explaining my protein biochemistry questions in a way I could understand.

Finally, my deepest thanks go to my best friend and husband Steve. I am grateful for his constant love and support, and for his ability to make me laugh even during the toughest of times.

TABLE OF CONTENTS

LIST OF FIGURES

Manuscript 1

Manuscript 2

LIST OF TABLES

Manuscript 1

Manuscript 2

LITERATURE REVIEW

Soybean Breeding and the *Prolina* Cultivar

The United States Department of Agriculture (USDA) began a soybean breeding program in the 1950's to increase the value of soybeans. Four main goals were set for this program: a) increase the nutritional quality for animal feed (e.g. increase sulfur-containing amino acid content), b) increase seed yield per plant, c) increase resistance of soybean plants to disease and pests, and d) break the negative correlation between protein, oil, and yield (*1*). Selective breeding spurred by this program has recently led to the development of the *Prolina* cultivar, which has an increased protein content without negatively affecting oil content or overall yield. Soybeans from this variety contain 46% protein (vs. 40% protein for conventional beans) and 19% oil (vs. 20% oil for conventional beans). *Prolina* also exhibits a 14% increase in essential amino acids per metric ton of soybeans (*2*).

The functionality of soy proteins from various cultivars may be influenced by such compositional differences as the ratio of protein fractions (particularly glycinin (11S)/β-conglycinin (7S)), variations in subunit concentrations within fractions, or differences in amino acid profiles (*3*). In addition to an increased content of storage protein, *Prolina* also has more cysteine residues (sulfur-containing amino acid) per mole of protein than other cultivars (*2*). This increase is most evident in the β-conglycinin fraction. Increased cysteine residues may enhance gelation properties by oxidizing to form disulfide bridges between protein molecules. These additionally improve nutritional characteristics when added to animal feeds (*2*).

Soy Proteins

Soy proteins are commonly classified into fractions by their sedimentation behavior using ultracentrifugation. This technique yields four fractions: 2S, 7S, 11S, and 15S, in which S stands for Svedberg unit.

The 2S fraction (8% of total protein) contains low molecular weight proteins, including trypsin inhibitors, cytochrome c, and other globulins (*4*). Trypsin inhibitors present in soy used for feeds would inhibit growth of animals if not inactivated by heat treatment. Because the 2S fraction is present in such small quantities and has limited functionality as a food ingredient, little research focuses on this component (*5*).

The storage proteins, β-conglycinin and glycinin, are the principal components of soy proteins and exhibit the most functionality (*6*). β-conglycinin is the chief constituent (~85%) of the 7S fraction, which makes up over one-third of the total protein. The remainder consists of lipoxygenases, hemagglutinins, and amylases; proteins in this fraction range in molecular weight from 141,000 to 171,000 daltons (*4*). β-conglycinin dimerizes when the ionic strength is lowered from 0.5 to 0.1, causing the ultracentrifugation peak to shift to 9S. The three major subunits of β-conglycinin (α, α′, and β) are associated by hydrophobic interactions to form trimeric proteins, of which 6 isomers have been isolated. The molecular weights are 57,000 for the α and α′ subunits and 42,000 for the β; their isoelectric points are 4.9, 5.2, and 5.7-6.0 respectively (*7*). Each subunit is glycosylated, containing 4-5% carbohydrates, mainly mannose and N-acetylglucosamine. In traditional soybean varieties, the β-conglycinin fraction contains fewer cysteines than glycinin (only 2-3 per mole of protein) (*4*). Therefore, disulfide

bonds are not formed during gelation of β-conglycinin.

Glycinin is the most abundant protein in soybeans, accounting for 52% of the 11S fraction and 5% of the 15S fractions, the latter existing as glycinin polymers. The 11S glycinin is a hexamer with a molecular weight varying from 320,000 to 363,000 daltons (*4*). Its quaternary structure is composed of 12 subunits, six acidic (35,000 daltons) and six basic (20,000 daltons), which are linked by disulfide bonds. The isoelectric point of glycinin is pH 4.64 (*8*). Unlike â-conglycinin, glycinin is sulfur-rich and contains 2-6 free sulfhydryls (*4, 9 –12*) and 20-37 disulfides per mole of protein (*4, 9, 11, 12*). Variations in reported cysteine contents may be attributed to differences between soybean cultivars, type of denaturants, conditions of solubilization, etc. (*9*). Intermolecular disulfide bonding has been found to be involved in gelation of glycinin (*13, 14*).

Individual investigation of 7S and 11S components has contributed to elucidating the heat-induced gelation properties of soy proteins. Their varying gelation properties primarily depend on heating temperature since β-conglycinin is less thermally stable than glycinin. The denaturation temperature (Td) of β-conglycinin, as measured by differential scanning calorimetry, ranges from 68-77 °C, while Td of glycinin ranges from 85-92 °C (*7, 15 - 19*). Variations in reported Td values may be due to soybean composition, subunit heterogeneity, sample preparation, pH, and heating rate. Glycinin thus plays a significant role in the gel formation of soy proteins at 100 ºC or above, while β-conglycinin is important in gel formation at 80 °C or above.

Interactions between the subunits of the 7S and 11S fractions occur during heating. When heated above their respective denaturation temperatures, the dissociated

basic subunits of the 11S interact electrostatically with the 7S subunits, forming soluble complexes (*20*). Nakamura et al. (*21*) concluded that the forces involved in the interactions among 7S and between 7S and 11S proteins are noncovalent (specifically hydrophobic interactions), while both noncovalent and disulfide bonds are involved in the interactions among 11S proteins alone.

Commercial Processing of Soy Proteins

Soy protein products have been widely used as food ingredients to enhance the value of finished foods. They are so versatile that they can now be found in virtually every food system. Primary soy protein ingredients include soy flours (48% protein), textured soy proteins, soy protein concentrates (70% protein), and soy protein isolates (90% protein) (*22*).

For processing, selected soybeans are cleaned to remove foreign material. The beans are dried, cracked, dehulled, conditioned, and rolled into flakes to increase surface exposure of their mass, enhancing subsequent defatting by hexane extraction. Residual hexane is removed in a vapor desolventizing system (VSD) or a flash desolventizing system (FDS). Defatted soy meal is produced by grinding the defatted, desolventized flakes; it contains over 50% protein, 30-35% carbohydrate, and less than 1% oil, with ash and moisture as the remaining components (*23*).

Soy flour is produced by grinding the defatted soy flakes into a fine powder. Soy flours are typically used in bakery products to improve color, nutrition, and moisture retention.

When higher protein contents are necessary, steps are taken to remove nonproteinaceous materials. Soy protein concentrates are prepared by removing the soluble carbohydrates and flavor compounds from defatted meal through aqueous alcohol extraction or an acid leaching process. Soy concentrates are utilized in a wide variety of food products including meats, bakery products and dairy analogs.

Soy flour and concentrates may be extruded under high temperature and pressure, resulting in fibrous textured soy proteins. Textured soy proteins are generally used as a meat replacer when a chewy texture is desirable.

Soy protein isolates are prepared from defatted soy meal using aqueous, alkali extraction (pH 7-10). Centrifugation of this protein slurry removes the insoluble residue, mainly carbohydrates. The soluble extract then adjusted to pH 4.5, which facilitates isoelectric precipitation of the soy protein. The precipitated protein is separated, washed, and spray-dried. Prior to spray drying, the precipitate may be neutralized (proteinate form) or left untreated (isoelectric form). The final product is highly purified (90% protein) and fairly bland in taste.

Although they are more expensive than soy concentrates, soy isolates exhibit improved flavor and color. Wang and Zayas (*24*) found that addition of soy concentrate and soy flour decreased the meaty flavor of frankfurters, while 2% soy isolate rated higher in meaty flavor compared to a meat only control. No significant differences in color were found between the all-meat control and samples containing soy isolates additives, while addition of soy flour or soy concentrates altered frankfurter color. Wang and Zayas (*24*) also studied the effect of soy protein addition on water holding capacity, cook loss, and texture of frankfurters. Meat batters extended with soy proteins had higher

water holding capacity and decreased cook loss than control samples, but no differences between isolates and concentrates were found, likely due to the presence of carbohydrates in soy concentrates. Textural characteristics, shear force and firmness were not affected by addition of either soy isolate or concentrate.

Processing soy proteins affects their biological activity; heat treatment is normally applied in conjunction with the desolventization step to inactivate lipoxygenase and trypsin inhibitors. In addition, protein solubility may be altered during oil extraction and subsequent heat processing. The extent of denaturation and loss of solubility depends on the intensity of heat treatment given to the product during processing (*22*). The designated pH during extraction, precipitation, and neutralization steps also influences final protein solubility (*25*). Protein insolubilization may also occur during drying as a result of protein polymerization through disulfide bonding and other protein-protein reactions (*26*).

Consequently, the most frequently used quality criterion for soy protein isolate used by industry is solubility, which is usually determined by the Protein Dispersibility Index (PDI) and the Nitrogen Solubility Index (NSI). PDI, or "fast stir" method is measured by water extraction of the proteins, centrifugation, and protein analysis of the supernatant and is expressed as percent water dispersible protein divided by percent total protein. NSI, or "slow stir" method measures water soluble nitrogen as a percentage of total nitrogen and is sometimes used as a measure of protein dispersibility. PDI and NSI were developed and adopted by the industry as a measure of the heat treatment of soy. A high PDI/NSI indicates that the proteins are very soluble whereas a low PDI/NSI implies greater heat treatment and thus lower protein solubility (*22*). But "quality" of soy isolates

is ultimately determined by how well they carry out the intended function in a given food application. Therefore highly denatured, poorly soluble isolates may be considered as high quality ingredients for specific applications. In addition, a single solubility measurement at a given pH and ionic strength is insufficient to characterize the solubility of a soy protein isolate (*27*). For example, soy proteins are largely insoluble at pH 4-5 and solubility increases at pH values outside this range. Therefore, most soy isolates are supplied as proteinates with a neutral pH to enhance their solubility.

The preceding commercial process is only a general description of soy protein isolate production. Several variations in one or more steps of this process may be performed to produce isolates with very specific properties, functions, and end uses. For example, during aqueous extraction the temperature, ratio of solids to solvent, pH, and use of alkaline reagents may vary. The drying step may involve spray drying, roller drying or freeze drying. Additional variations are outlined in the following patented processes. A process for preparing thermoreversible gels from isolates (*28*) involves treatment of the acid precipitated fraction of an aqueous whole extract of hexane-extracted soybean meal with certain critical ranges of lower alcohols (methanol, ethanol, and isopropanol). During this treatment, an anti-gelling factor is reportedly removed. Anson and Pader (*29*) prepared soy isolates for gel-like meat products. Isolates were adjusted to pH 6-7.5 and 14-35% solids. This gel precursor (semi-solid paste) was then heat treated with steam. Using the resulting gel, protein binders could be prepared and cooked to form simulated meats (*30*).

Soy Protein Isolate Functionality in Comminuted Meat Products

While soy proteins certainly contribute nutritional benefits, they are utilized as food ingredients primarily for their functional contributions including water holding, fat binding, emulsification, and gelation. These particular functional properties have led to extensive use of soy protein isolates in comminuted meat products (frankfurters, bologna, meatballs, luncheon meats, etc.).

Hydrophilic residues allow soy protein isolates to absorb and retain water. Terms used to describe the spontaneous uptake of water include water absorption capacity or water imbibing capacity. Protein-water interactions describing ability to physically hold water during the application of forces (gravity, centrifugation, heating) have been expressed interchangeably using the terms water holding, water binding, and water retention (*26*). Water holding varies with protein source, composition, the presence of carbohydrates and lipids, pH, and salts. Water holding capacity of a soy protein preparation increases with increasing protein content so soy isolates hold more water than soy concentrates (*23*). Water binding of soy protein isolate is very dependent on pH as increasing from pH 5 to pH 7 (moving away from the isoelectric point) results in water binding capacity increases of nearly 100 times (*31*), while addition of 0.2 M NaCl decreased water absorbing capacity (*32*).

In comminuted meat products, soy isolates act as a water binder not only during product preparation, but also during cooking of the product as evidenced by increased water holding capacity (of meat batter) and decreased cooking loss (final product) compared to an all-meat control (*24, 33*). The functionality of soy protein isolates in comminuted meat products greatly depends on how the ingredient is added. Addition of

a soy protein dispersion vs. soy protein powder resulted in a significant decrease in cook loss (*7*). Overall, most data implies that soy protein isolates function by both absorbing (like a sponge) and retaining (physical entrapment) water during meat processing.

Fat binding is closely related to protein content, and more specifically, the hydrophobic regions of the protein. Therefore, fat binding is generally enhanced by protein denaturation. Fat binding is usually measured by adding an excess of oil to the protein product followed by mixing, centrifugation, and determination of amount of absorbed oil by weight (*31*). Due to their excellent water and fat binding properties, incorporation of soy protein isolates into comminuted meat products also enhances emulsion capacity and stabilizing emulsions. Soy proteins play two roles in emulsification; they aid in the formation of oil-in-water emulsions (emulsifying capacity) and stabilize the emulsions once they are formed (emulsifying stability).

When soy protein isolates are fully hydrated prior to its addition to meats, the gel matrix can immobilize fat particles and oil droplets and increase stability of the heat stable meat emulsion. Lecomte and Zayas (33) found that addition of 2% soy isolate (pre-emulsified) to sausage batters resulted in significantly less fat separation when compared to the all-meat control. The addition of 2% soy isolate (powder) also decreased fat separation compared to the control, but was not as effective as the pre-emulsified soy isolates.

Soy protein isolates have the ability to form gels which can act as a matrix for entrapping water, lipids, sugars, flavors, and other ingredients. Basic factors influencing soy protein gelation include protein concentration, heating temperature and time, ionic strength, and pH. In meat systems, the ability of commercially prepared soy isolates to

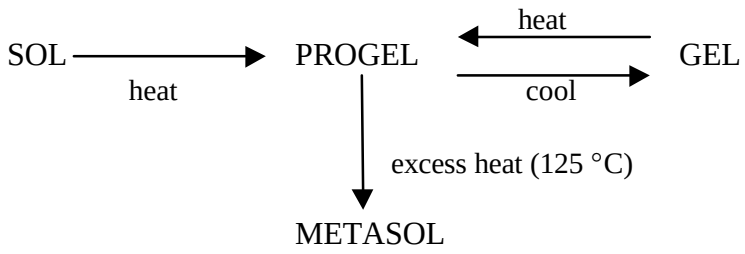
form gels below 80°C is an important property because a high temperature negatively affects the texture of meat products (*34*).

Soy Protein Gelation

Protein gelation involves development of a three-dimensional network stabilized by protein-protein and protein-solvent interactions. Gel formation usually proceeds upon heating with the denaturation of the native protein to a disordered arrangement, followed by an ordered aggregation of the denatured molecules and stabilization of the gel matrix with covalent (disulfide bonding) and noncovalent (hydrogen bonding, hydrophobic interactions, and electrostatic interactions) interactions (*35*). Whether a gel forms or not depends on the extent of crosslinking of the initial molecules present, and this in turn is influenced by environmental conditions, concentration, temperature, and heating time (*36*). Soy proteins form gels at concentrations above 7% protein (*37, 38*). At neutral pH, gels form within 10 – 30 min at 70 to 100 °C (*39*).

The majority of studies on soy protein gelation have focused on 'native' soy isolates carefully produced in the laboratory to be minimally denatured prior to characterization. In contrast, many commercially prepared soy protein isolates are intentionally denatured to some degree to improve their functionality for specific food applications. Such predenatured isolates behave quite differently from native soy isolates since their processing conditions have caused the proteins to assume various conformations and states of aggregation (*7*). In addition, gelation mechanisms and resulting gel structures and gel properties will differ between commercial and native soy protein gels (*40*).

An overall scheme for the gelation of soy proteins was proposed by Catsimpoolas and Meyer (*38*):



The protein dispersion (sol) is converted to a progel by heat (80 °C for 30 min), and upon cooling (to 4 °C) set to a gel. The progel state is characterized by high viscosity, due to protein unfolding. The sol to progel transition is irreversible, while the progel to gel transition was considered reversible. The bonds involved in gel formation are likely primarily noncovalent, since gels apparently "melted" at temperatures as low as 40°C (*38*). It is noted however, that physical (temperature at which a steel ball placed on the gel reached the bottom of a tube), rather than rheological measurements determined melting of gels. The possibility exists that the progel state observed by Catsimpoolas and Meyer (*38*) may have actually been a weak gel. Belitz and Grosch (*41*) noted that, while thermally irreversible gels do not liquefy when heated, they may soften or shrink. With excess heat (125 °C), the progel formed what Catsimpoolas and Meyer (*38*) termed a metasol, which did not gel upon cooling. This conversion was accompanied by chemical degradation of the proteins, observed as a release of ammonia and decomposition of cystine. Excessive heating at high temperatures (>100 °C) often causes scission of primary peptide linkages in proteins, preventing gel network formation (*42*). A metasol was also obtained in the presence of reducing agents or denaturants (*38*).

Several researchers (*7, 18, 40, 43, 44*) have noted that certain commercially prepared (relatively highly denatured) soy protein isolates do not exhibit a "progel state". In native soy protein isolates, gel rigidity increases after the onset of denaturation of β-conglycinin and progressively increases during further heating, holding, and cooling (*19, 45, 46*). In contrast, these more denatured soy isolates, at sufficient protein concentration and ideal pH, ionic strength, etc., readily gel upon hydration at room temperature (*18, 43, 44*). Upon further heating, gel rigidity decreases, which has been attributed to weakened hydrogen bonding. However, subsequent cooling reinforces the gel network by restoring extensive hydrogen bonding.

Luck et al. (*44*) found that during holding of commercial soy isolate pastes at 90 °C for 30 min, structure formation occurred which suggested possible contribution of intermolecular hydrophobic interactions and/or disulfide bonding. Renkema and van Vliet (*19*) observed an increase in gel rigidity during prolonged heating of native soy isolates at 90 °C. They attributed this to increased protein incorporation into the gel network leading to further strengthening and/or due to rearrangements occurring in the network. Luck et al. (*44*) also found that, after cooling, gel rigidity was higher than prior to heating, further indicating formation of thermally irreversible bonds (hydrophobic and/or covalent) during the heating/holding treatment. In contrast, other researchers (*18, 43*) observed no changes during holding of heated commercial soy isolate gels, and heated and cooled gels reached a G′ value (indicative of gel rigidity) lower than that measured prior to heating.

Due to varied processing conditions, soybean composition, and levels of denaturation, differences in gelation properties of commercially prepared soy protein

isolates are likely. Surfactants are also sometimes added to improve solubility/dispersibility for beverage or comminuted meat product applications (*4*), thus allowing the soy proteins to interact more readily with water.

Heating temperature plays a crucial role in texture development of soy protein gels due to the contribution of particular molecular forces (hydrogen bonding, hydrophobic interactions, and disulfide bonding) at varying temperature ranges and the extent of denaturation induced in the proteins. Even though many commercially prepared soy isolates have already experienced extensive protein denaturation, gel-forming ability is altered upon further thermal treatment. Partially denatured commercial soy isolates may be further denatured upon additional heat treatment, providing that denaturation temperatures are reached.

Furukawa et al. (*47*) subjectively classified 20% commercial soy isolate gels formed by holding 30 min at temperatures ranging from 25 °C to 130 °C and subsequently cooled into three groups: “soft”, “hard”, and “fragile”. Heating below 50 °C resulted in “soft” gel formation, while heating at temperatures from 60 °C to 110 °C induced formation of “hard” gels. “Fragile” gels resulted from heating at temperatures greater than 120 °C, which corresponds to the metasol state of Catsimpoolas and Meyer (*38*). Gel hardness, measured at each elevated temperature, increased linearly with heating temperature up to 80 °C, which they attributed to increased hydrophobic interactions. Gel hardness might also increase upon heating due to increasing formation of intermolecular disulfide bonds in the gel matrix (*48*). Shimada and Cheftel (*9*) found no change in gel firmness of 16% commercially prepared soy isolate gels when the heating temperature (for 30 min) was increased from 80 to 105 °C. However a marked

increase in gel firmness was apparent when the soy dispersion was heated at 115 or 120 °C, followed by a drastic decrease in gel firmness when heated at 130 °C. The authors believed that association takes place via both noncovalent interactions and formation of intermolecular disulfide bonds at temperatures up to 120 °C, but at higher temperatures degradation of the gel network takes place. The latter they attributed to breakdown of disulfide bonds as evidenced by an increase in free sulfhydryl groups.

Even though soy proteins contain several cysteine residues researchers have disagreed on the extent of intermolecular disulfide bonding that occurs in soy protein gels. Catsimpoolas and Meyer (*38*) concluded that covalent crosslinks are not involved in gel formation because gels melted at 40 °C. However, Circle et al. (*37*) considered covalent crosslink formation an important mechanism in soy protein gel formation due to the inhibitory effect of disulfide reducing agents on gelation. Catsimpoolas and Meyer (*38*) only heated protein dispersions at 80 °C for 30 min, which most likely only denatured the β-conglycinin fraction. Gelation of β-conglycinin involves hydrogen bonding and hydrophobic interactions and gels formed from this protein fraction alone are thermally reversible (*49*). Circle et al. (*37*) heated the native soy proteins at 100 °C for 30 min, which would have denatured both the β-conglycinin and glycinin fractions. Gelation of glycinin involves disulfide bonding in addition to hydrogen bonding and hydrophobic interactions, resulting in thermally irreversible gels (*13, 14*).

Therefore, a more native soy protein isolate heated to 80 ºC should form thermally reversible gels, whereas heating native soy proteins to 100 ºC should result in formation of thermally irreversible gels. No gelation model has been proposed for

commercially prepared soy protein isolates, but formation of thermally irreversible gels (even at 80 ºC) would be likely since the proteins would have been previously denatured.

Molecular Forces Involved in Soy Protein Gelation

Protein-protein interactions responsible for gel formation may be noncovalent (hydrogen bonds, electrostatic interactions, and hydrophobic interactions) or covalent (disulfide bonds). Environmental conditions such as pH, ionic strength, temperature, and presence of thiol/disulfide reagents or denaturants will affect the availability of these bonding groups for stabilization of tertiary structure and intermolecular interactions. It is important to understand the mechanisms involved and the contribution of molecular forces in soy protein gelation in order to control gel properties.

Hydrogen Bonds

Hydrogen bonds involve the interaction of a hydrogen atom that is covalently attached to an electronegative atom (i.e. nitrogen, oxygen, or sulfur) and electrostatically interacts with another electronegative atom (*50*). Hydrogen bonds are weak relative to other interactions, but large numbers of such interactions can lead to gel formation. In proteins, the α-helix and β-sheet structures are stabilized by hydrogen bonding. At low temperatures the protein remains a helix, but with increasing temperature the conformation changes to a random coil (i.e. denatures) thereby disrupting hydrogen bonds (*51*). Intermolecular hydrogen bonding is largely reversible upon cooling as secondary structures are partially regained.

Renkema and van Vliet (*19*) studied the thermal reversibility of the maximum gel rigidity (G′) attained upon cooling of heated 11% native soy isolate gels. When this gel

was reheated to 90 °C, the G′ curves overlapped. This increase in G′ during cooling was obviously due to reversible formation of hydrogen bonds.

Electrostatic Interactions

Electrostatic interactions become important when investigating pH and/or ionic strength effects. While the pH determines the total charge of the protein, the ionic strength and ion types determine the extent of interaction among those charges since salts can shield charges (*51*). Proteins assume either a net negative or a net positive charge at neutral pH, depending on the relative number of negatively (aspartic and glutamic acids) and positively charged (arginine, lysine, and histidine) amino acid residues. Most of the charged groups are distributed on the surface of the protein molecule. The net repulsive interaction between like charges could possibly destabilize gel structure while attractive interactions between oppositely charged groups might contribute to gel stability (*50*).

Hydrophobic Interactions

Dissociation and/or unfolding of soy protein molecules increases the exposure of interior nonpolar amino acids (alanine, valine, leucine, isoleucine, proline, phenylalanine, tryptophan, tyrosine, methionine), promoting protein-protein hydrophobic interactions. When two hydrophobic molecules come into contact, the surrounding water is displaced ("the hydrophobic effect"). The repulsive behavior of nonpolar solutes for water becomes less entropy driven at higher temperatures (*51*). Opposing exothermic hydrogen bonding, hydrophobic interactions are endothermic so they are enhanced at high temperatures (the free energy change becomes more negative) and weakened at lower temperatures. As temperature is lowered, the water becomes more extensively hydrogen bonded, decreasing stabilization of hydrophobic interactions. Their strength typically

reaches a maximum at about 60-70 ºC depending on the protein (*50*). Above a certain temperature, gradual breakdown of hydrogen-bonded water structure will eventually destabilize hydrophobic interactions (*50*).

Disulfide Bonds

Disulfide bonds are the only covalent protein-protein crosslinks naturally formed in soy proteins and are created through thiol-disulfide exchange and/or sulfhydryl oxidation. Thiol-disulfide interchange involves a reaction between an ionized thiol group, the thiolate anion (nucleophile), and a disulfide bond (*52*). The rate of the reaction generally increases as the pH is increased, until the pKa of the reactive group (typically cysteine; pKa 9.0-9.5) is reached. Sulfhydryl oxidation occurs when free sulfhydryls react to form cystine (S-S) in the presence of molecular oxygen.

Heating proteins may unmask internally buried sulfhydryl (SH) groups and promotes their oxidation to disulfide bonds (SS). Heating also promotes the exchange of intramolecular disulfide bonds to intermolecular bonds that can add to gel strength. The presence of a large number of SH and SS groups in soy proteins can thus strengthen the protein intermolecular network and contribute to thermal irreversibility. Shimada and Cheftel (*9*) thoroughly investigated the changes in SH groups and SS bond contents in a commercial soy protein isolate during heat-induced gelation. In unheated soy protein isolate, total SH groups and free SH groups were 8.0 and 5.0 μmol/g of total protein respectively. Heating at 80 °C for 30 min reduced the content of total SH groups but had no significant effect on the free SH group content. These researchers suggested that decrease in total SH groups is due to oxidation into additional SS bonds, since the half-cystine content remained constant.

Rheological Properties of Gels

The gel state may be defined by the ability to immobilize a liquid, as a continuous macromolecular structure, or by textural or rheological properties (*35*). Rheologically, gels have been defined as "substantially diluted systems which exhibit no steady state flow" (*53*). Gels exhibit a combination of both viscous and elastic behavior, and thus are considered viscoelastic materials. Rheological properties of viscoelastic materials can be evaluated by either small strain (deformation) or failure/fracture tests; a complete rheological evaluation would include both methods.

Dynamic, small strain rheological measurements can be used to study the progression of a protein suspension from the sol to gel state since it is sensitive to rheological properties of both the sol and gel (and all intermediate states) and it does not appreciably disturb the structure of the gel being formed. Stress or strain sweeps are performed (on sol and/or gel) to ensure that measurements are made within the linear viscoelastic region (where stress $\propto$ strain), defined as the region within which no structural damage to the material is occurring.

Evaluation of the shear stress response to small amplitude oscillatory (usually sinusoidal) deformation results in evaluation of two primary rheological parameters. G' (storage modulus) is a measure of the energy stored by the gel during deformation and indicates the elastic (rigidity) component of the gel. G'' (loss modulus) is a measure of the energy lost in the recovery from deformation and reveals the viscous nature of the gel. The sinusoidally varying shear stress or shear strain responses are separated by a phase lag. The ratio of G'' to G' is equal to the tangent of the phase angle (δ) of this lag. As the

gel network is formed, the material becomes more elastic and so G′ values increase, while the tan δ decreases.

The moduli (G′ and G″) can also be measured as a function of frequency. This common form of testing shows how the elastic and viscous behavior of the material changes with the rate of application of stress or strain. As the frequency increases, elastic behavior will predominate. When the sol becomes a gel, G′ > G″, and plots of G′ and G″ vs. frequency are parallel.

Large deformation or fracture testing of gels best correlates to the sensory evaluation of gel texture (*35, 54*). Torsion (twisting) and uniaxial compression (applying a force perpendicular to the sample) are most commonly used to deform the sample to the point of structural failure (*55*). Both methods may be used to assess fundamental values of shear stress and shear strain at fracture; stress reflects the hardness or firmness of the gel and strain is related to deformability or cohesiveness. The torsion method is best applied to stronger, more deformable gels and compression for weaker gels that cannot be easily tested by torsion (*55*). The vane method offers an alternative to torsion and compression testing (*56*). A vane with 4 - 8 blades is immersed into a gel and slowly rotated, while the gel is held fixed, at a constant rate until gel fracture occurs. The maximum torque and time to fracture is used to calculate stress and deformation values.

Common alternative testing methods for fracture testing are by penetration (punch test) or Instron texture profile analysis (force to fracture, hardness, etc.). Such empirical methods are dependent on sample size and shape, while more fundamental determination of shear stress and strain (through compression, torsion, or vane method) give a more complete textural evaluation. For example, a soft deformable gel may have the same

failure stress (hardness) as a rigid brittle gel; however, the failure strain (deformation) could vary greatly between the samples (*57*).

Gel rheological properties, such as rigidity, hardness, and deformability can vary widely depending upon factors such as protein concentration, pH, ionic strength, and heating time/temperature. Nakamura and others (*21*) found that hardness of soy protein gels increased sigmoidally with protein concentration over the range of 2.5 – 20%. Increased protein concentration enhances probability of protein-protein interactions, which in turn contribute to gel strength. Shimada and Cheftel (*9*) also found that firmness of soy protein gels increased exponentially with increasing protein concentration (0.9 – 18.2%). They also found that gel firmness linearly increased with increasing pH (7 – 10); the firmness at pH 10 was approximately 5 times greater than firmness at pH 7 and was attributed to additional formation of disulfide bonds at alkaline pH (disulfide bond formation requires S^-). Puppo and Anon (*58*) found that, as the pH of soy gels was increased, fracture strength increased and strain (deformability) decreased. This effect was attributed to greater formation of disulfide bonds at alkaline pH. Utsumi and Kinsella (*13*) found that hardness of soy gels decreased with increasing ionic strength (0 – 0.5 M NaCl). NaCl is known to stabilize protein structure (*7*) so it is likely that at the applied temperatures adequate denaturation was not achieved, which is necessary for protein aggregation reactions to take place.

Elucidating Bonding Types and Contribution

One approach to assessing the bonding forces involved in gel formation is through addition of particular chemical modifiers (salts, disulfide/thiol reagents, or denaturants)

prior to heating and observing their effect on the gelation process using rheological analyses (both small and large strain). Such reagents may also be added after gel formation to determine whether the gel can be solubilized by weakening specific intermolecular bonds.

Addition of neutral salts may aid in explaining contribution of major noncovalent forces involved in the gelation of soy protein isolate. At low concentrations, salts tend to neutralize electrostatic interactions between charged groups in a protein, whereas at higher concentrations salts have ion-specific effects on hydrophobic interactions (*16*). The effectiveness of various salts on protein stability follows the Hofmeister series for anions, $F^->SO_4^{2-}>Cl^->I^->ClO_4^->SCN^-$ (*16, 59*). Therefore, sodium chloride (NaCl) and sodium bisulfite (Na_2SO_4) act as structure stabilizers whereas sodium thiocyanate (NaSCN) and sodium chlorite ($NaClO_4$) act as destabilizers of hydrophobic interactions. The mechanism by which particular ions affect the structural stability of proteins is not well understood but likely involves their relative abilities to bind to and alter hydration properties (*50*). Salts that stabilize proteins enhance hydration of proteins (and hydrogen bonding) and bind weakly, whereas salts that destabilize proteins decrease protein hydration and bind strongly (*50*).

Using dynamic rheology, Nagano et al. (*49*) studied the effects of various concentrations of NaSCN and NaCl on 15% 7S globulin gelation (80 °C for 30 min, cooled to 20 °C). In the presence of 1 M NaSCN or with increasing concentrations of NaCl , the normal increase in G′ was inhibited during the heating process. The action of NaSCN was attributed to destabilization of hydrophobic interactions while the effects of NaCl were credited to the stabilizing effect of NaCl against denaturation. Since

hydrophobic interactions are enhanced at higher temperatures, G′ should have increased, but was suppressed by addition of NaSCN. NaCl has been previously found to increase the Td of soy proteins (*16*). Thus, as NaCl concentration was increased, the number of functional groups available for gel network formation may have been reduced due to insufficient denaturation of 7S globulin.

Addition of denaturants can be used to distinguish between formation of noncovalent and covalent bonds during gelation. In the presence of a denaturant such as urea, protein molecules exhibit little, if any, intra- and intermolecular noncovalent interactions. Therefore, if a protein paste containing urea or other denaturant exhibits thermally induced gelation it is clear that the formation of covalent crosslinks is involved in gelation (*60*).

Urea and guanidine hydrochloride (GuHCl) are denaturants due to their preferential interaction with proteins and excellent hydrogen bonding capability. Their addition to protein solutions results in breakdown of the hydrogen-bonded structure of water. Upon this disruption of hydrogen-bonded water, exposure of interior nonpolar groups allows unfolding and solubilization of nonpolar groups through hydrophobic interactions (*50*). Essentially, these denaturants displace water by binding to the protein, thus weakening hydrophobic interactions and hydrogen bonds between proteins (thus unfolding the protein). Urea or GuHCl-induced denaturation can be reversed through removal of the denaturant. GuHCl acts similarly to urea although it is more reactive as the unfolding process is significant in concentrations greater than 1 M GuHCl (vs. 4 M for urea) (*61*). Interestingly, many globular proteins do not undergo complete

denaturation even in 8 M urea, whereas in 6 M GuHCl, they usually exist in a random coil (*50*).

Nagano et al. (*49*) studied the effect of GuHCl on 15% 7S globulin gelation. With increasing concentration (0 - 4 M GuHCl), the G′ increase was inhibited during heating and no gel formed in more than 4 M GuHCl. This indicated the importance of hydrogen bonds and/or hydrophobic interactions to the gelation of 7S globulin, and suggested that contribution of intermolecular disulfide bonds is limited. Van Kleef (*45*) found that when a 15% soy isolate dispersion in 6 M urea solution was heated, no irreversible increase of G′ was seen (the heating and cooling curves overlapped). In addition, gels prepared in distilled water readily dissolved in the urea solution, indicating that noncovalent interactions were mainly involved in their gel formation. Several other researchers reported that addition of urea prevented gelation of soy protein isolates (*38, 45, 47*). However, soy isolate gels exhibited an initial G′ value > 100 Pa (*45*) and a notable gel hardness value (*47*); data was not shown in the paper from Catsimpoolas and Meyer (*38*). Obviously, gels were indeed formed in 6 M urea solutions and a more accurate explanation would be that urea addition either resulted in decreased gel strength or impaired the gelation process.

Several studies have utilized disulfide/thiol reagents to determine the effect of disulfide bonds on structural and functional properties of soy proteins. Addition of excess molar quantity of the reagents n-ethylmaleamide (NEM) (*9*), 2-mercaptoethanol (ME) (*13, 14, 47*), cysteine and sodium sulfite (*36*), and dithiothreitol (DTT) (*13*) drastically reduced hardness of soy protein gels. Cysteine and NEM react directly with

free SH groups, thus preventing disulfide bonding while DTT and ME reduce disulfide bonds to SH groups (*52*).

Utsumi and Kinsella (*13*) studied the effects of reducing agents on soy isolate gels heated at 80 °C for 30 min. In the presence of ME or DTT, fragile gels were formed and their hardness decreased up to a concentration of 0.01 M, but gel hardness increased above 0.02 M. These results were attributed to cleavage of intermolecular (at low concentrations) and intramolecular (at high concentrations) disulfide bonds. Cleavage of intramolecular disulfide bonds may facilitate exposure of interior functional groups, resulting in increased protein-protein interactions and thus enhancing gel strength. They also investigated the effect of 0.2 M ME and 8 M urea on the solubilization of 12% soy protein isolate gels. Approximately 47% of protein was solubilized by buffer and almost all the protein was solubilized by buffer containing either ME or urea.

Several researchers also reported that addition of disulfide reducing agents prevented gelation of soy protein isolates (*37*, *38*, *47*). However, Furukawa et al. (*47*) actually found that addition of 0.01 ME to 20% soy protein isolates resulted in weak, fragile gels whereas in the case of Circle et al. (*37*) gelation of 10% soy isolates was prevented by addition of sodium sulfite or cysteine. These differences reflect the importance of protein concentration on formation of soy isolate gel networks. Also, in comparing the effects of denaturants vs. disulfide/thiol reagents it seems that the latter has a more detrimental effect on final gel strength. Perhaps this is a result of the varying effectiveness of each reagent type. For example, noncovalent interactions are the dominant forces in protein folding and at high protein concentrations even concentrated urea solutions may not be successful in suppressing all of these interactions. On the other

hand, the extent of disulfide bonding is likely lower overall and may be easily blocked by small quantities of disulfide or thiol reagents.

The majority of researchers (*9, 13, 14, 21, 47*) have concluded that soy protein gel networks are formed through a combination of forces – hydrogen bonding, hydrophobic interactions, and disulfide bonding, although the extent of each force is subject to debate. Clearly, these conclusions have been supported by research involving addition of chemical modifiers or neutral salts, pH effects, and various thermal treatments and through subsequent evaluation of resulting gels. Variations in preparation and processing of soy protein isolates, ratio of glycinin to β-conglycinin, and amino acid composition also must be considered when studying gel properties. Further research is necessary to examine the contribution of each of these individual forces in each stage of soy protein gel formation.

LITERATURE CITED

(1) Luck, P.J. Gelling properties of isolate from a high protein soybean cultivar and its functionality in a comminuted meat system [MSc thesis]. Raleigh, NC: North Carolina State University. 2000; 70 p. Available from: DH Hill Library, Raleigh, NC.

(2) Kwanyuen, P.; Wilson, R.F.; Burton, J.W. Soybean Protein Quality. In *Emerging Technologies, Current Practices, Quality Control, Technology Transfer, and Environmental Issues*; Koseoglu, S.S., Rhee K.C., Wilson, R.F., Eds.; Volume I in The Proceedings of the World Conference on Oilseed and Edible Oils Processing. Champaign, IL, 1998; pp 284-289.

(3) Riblett, A.L.; Herald, T.J.; Schmidt, K.A.; Tilley, K.A. Characterization of β-conglycinin and glycinin soy protein fractions from four selected soybean genotypes. *J. Agric. Food Chem.* **2001**, *49*, 4983-4989.

(4) Kinsella, J.E. Functional properties of soy proteins. *J. Amer. Oil Chem. Soc.* **1979**, *56*, 242-258.

(5) Liu, K. *Soybeans: Chemistry, Technology, and Utilization*; Chapman & Hall: New York, 1997.

(6) Morr, C.V. Current status of soy protein functionality in food systems. *J. Amer. Oil Chem. Soc.* **1990**, *67*, 265-271.

(7) Hermansson, A.-M. Soy protein gelation. *J. Amer. Oil Chem. Soc.* **1986**, *63*, 658-666.

(8) Snyder H.E.; Kwon, T.W. *Soybean Utilization*; Van Nostrand Reinhold Company, Inc.: New York, 1987.

(9) Shimada K.; Cheftel, J;C. Determination of sulfhydryl groups and disulfide bonds in heat-induced gels of soy protein isolate. *J. Agric. Food Chem.* **1988**, *36*, 147-153.

(10) Wolf, W.J. Sulfhydryl content of glycinin: effect of reducing agents. *J. Agric. Food Chem.* **1993**, *41*, 168-176.

(11) Badley, R.A.; Atkinson, D.; Hauser, H; Oldani, D.; Green, J.P.; Stubbs, J.M. The structure, physical, and chemical properties of the soybean protein glycinin. *Biochimica. et Biophysica. Acta.* **1975**, *412*, 214-228.

(12) Draper, M.; Catsimpoolas, N. Disulfide and sulfhydryl groups in glycinin. Cereal Chem. **1978**, *55*, 16-23.

(13) Utsumi, S.; Kinsella, J.E. Forces involved in soy protein gelation: effects of various reagents on the formation, hardness, and solubility of heat-induced gels made from 7S, 11S, and soy isolate. *J. Food Sci.* **1985**, *50*, 1278-1282.

(14) Mori, T.; Nakamura, T.; Utsumi, S. Behavior of intermolecular bond formation in the late stage of heat-induced gelation of glycinin. *J. Agric. Food Chem.* **1986**, *34*, 33-36.

(15) Hermansson, A.-M. Physico-chemical aspects of soy protein structure formation. *J. Texture Stud.* **1978**, *9*, 33-58.

(16) Damodaran, S. Refolding of thermally unfolded soy proteins during the cooling regime of the gelation process: effect on gelation. *J. Agric. Food Chem.* **1988**, *36*, 262-269.

(17) Nagano, T.; Hirotsuka, M.; Mori, H.; Kohyama, K.; Nishinari, K. Dynamic viscoelastic study on the gelation of 7S globulin from soybeans. *J. Agric. Food Chem*. **1992**, *40*, 941-944.

(18) Chronakis, I.S.; Kasapis, S.; Richardson, R.K.; Doxastakis, G. Characterisation of a commercial soy isolate by physical techniques. *J. Texture Stud.* **1995**, *26*, 371-389.

(19) Renkema, J.M.S.; van Vliet, T. Heat-induced gel formation of soy protein at neutral pH. *J. Agric. Food Chem.* **2002**, *50*, 1569-1573.

(20) Damodaran, S.; Kinsella, J.E. Effect of conglycinin on the thermal aggregation of glycinin. *J. Agric. Food Chem.* **1982**, *30*, 812-817.

(21) Nakamura, T.; Utsumi, S.; Mori, T. Interactions during heat-induced gelation in a mixed system of soybean 7S and 11S globulins. *Agric. Biol. Chem.* **1986**, *50*, 2429-2435.

(22) de Meester, J.; Kempener, S.; Mollee, P. Production and isolation of soy proteins. *Industrial Proteins* **2000**, *8*, 5-7.

(23) Hettiarachchy, N.; Kalapathy, U. Soybean protein products. In *Soybeans: chemistry, technology, and utilization*; Liu, K, Ed.; Chapman & Hall: New York, 1997; pp 379-407.

(24) Wang, C.H.; Zayas, J.F. Comparative study of corn germ and soy proteins utilized in comminuted meat products. *Food Quality* **1992**, 15,153-167.

(25) Lillford, P.J. Extraction processes and their effect on protein functionality. *Qual. Plant Plant Hum Nutr*. **1983**, *32*, 401-409.

(26) Zayas, J.F. *Functionality of proteins in food*. Springer: New York, 1997.

(27) Shen, J.L. Solubility profile, intrinsic viscosity, and optical rotation studies of acid precipitated soy protein and of commercial soy isolate. *J. Agric. Food Chem.* **1976**, *24*, 784-788.

(28) Eldridge, A.C.; Nash, A.M. U.S. Patent 3,218,307; November 16, 1965

(29) Anson, M.L.; Pader, M. U.S. Patent 2,830,902; November 12, 1957.

(30) Hanson, L.P. *Vegetable protein processing*; Noyes Data Corporation: Park Ridge, NJ, 1974.

(31) Visser, A.; Thomas, A. Review: soya protein products – their processing, functionality, and application aspects. *Food. Rev. Int*. **1987**, *3*, 1-32.

(32) Wagner, J.R.; Anon, M.C. Influence of denaturation, hydrophobicity and sulfhydryl content on solubility and water absorbing capacity of soy protein isolates. *J. Food Sci*. **1990**, *55*, 765-770.

(33) Lecomte, N.B.; Zayas, J.F. Properties of batters and storage stability of frankfurters containing preemulsified fat stabilized with soy proteins. *J. Food Proc. Preserv.* **1993**, *17*, 287-304.

(34) Nagano, T.; Fukuda, Y.; Akasaka, T. Dynamic viscoelastic study on the gelation properties of β-conglycinin-rich and glycinin-rich soybean protein isolates. *J. Agric. Food Chem.* **1996**, *44*, 3484-3488.

(35) Ziegler, G.R.; Foegeding, E.A. The gelation of proteins. In *Advances in Food And Nutrition Research Vol. 34*; Kinsella, J.E., Ed.; Academic Press, Inc.: New York, 1990; pp 203-298.

(36) Clark, A.H. Gelation of globular proteins. In *Functional Properties of Food Macromolecules*; Hill, S.E., Ledward, D.A., Mitchell, J.R., Eds.; Chapman & Hall: Gaithersburg, Maryland, 1998; pp77-143.

(37) Circle, S.J.; Meyer, E.W.; Whitney, R.W. Rheology of soy protein dispersions. Effect of heat and other factors on gelation. *Cereal Chem.* **1964**, *41*, 157-172.

(38) Catsimpoolas, N.; Meyer, E.W. Gelation phenomena of soybean globulins. I. Protein-protein interactions. *Cereal Chem.* **1970**, *47*, 559-570.

(39) Wolf, W.J. Soybean proteins: their functional, chemical, and physical properties. *J. Agric. Food Chem.* **1970**, *18*, 969-976.

(40) Hermansson, A.-M. Methods of studying functional characteristics of vegetable proteins. *J. Amer. Oil Chem. Soc*. **1979**, *56*, 272-279.

(41) Belitz, H.-D.; Grosch, W. *Food Chemistry*; Springer: New York, 1999.

(42) Damodaran, S. Structure-function relationship of food proteins. In *Protein Functionality in Food Systems*; Hettiarachchy, N.S., Ziegler, G.R., Eds.; Marcel Dekker, Inc.: New York, 1994; pp 1-37.

(43) Chronakis, I.S. Network formation and viscoelastic properties of commercial soy protein dispersions: effect of heat treatment, pH, and calcium ions. *Food-res-int* **1996**, *29*, 123-134.

(44) Luck, P.J.; Lanier, T.C.; Daubert, C.R.; Kwanyuen, P. Viscoelastic behavior of commercially processed soy isolate pastes during heating and cooling. *J. Food Sci.* **2002**, *67*, 1379-1382.

(45) Van Kleef, F.S.M. Thermally induced protein gelation: gelation and rheological characterization of highly concentrated ovalbumin and soybean protein gels. *Biopolymers* **1986**, *25*, 31-59.

(46) Owen, S.R.; Tung, M.A.; Paulson, A.T. Thermorheological studies of food polymer dispersions. *J. Food Eng.* **1992**, *16*, 39-53.

(47) Furukawa, T.; Ohta, S.; Yamamoto, A. Texture-structure relationships in heat-induced soy protein gels. *J. Texture Stud*. **1979**, *10*, 333-346.

(48) Furukawa, T.; Ohta, S. Mechanical and water-holding properties of heat-induced soy protein gels as related to their structural aspects. *J. Texture Stud.* **1982**, *13*, 59-69.

(49) Nagano, T.; Akasaka, T.; Nishinari, K. Effect of heating and cooling on the gelation kinetics of 7S globulin from soybeans. *J. Agric. Food Chem.* **1994**, *42*, 1415-1419.

(50) Damodaran, S. Amino acids, peptides, and proteins. In *Food Chemistry, 3rd ed*.; Fennema, O.R., Ed.; W.H. Freeman and Co.: New York, 1996; 321-430.

(51) Dill, K.A. Dominant forces in protein folding. *Biochemistry* **1990**, *29*, 7133-7155.

(52) Creighton, T.E. *Proteins – Structures and Molecular Properties, 2nd ed*.; W.H. Freeman and Co., 1993.

(53) Ferry, J.D. *Viscoelastic properties of polymers*; New York: Wiley, 1980

(54) Mitchell J.R. The rheology of gels. *J. Texture Stud.* **1976**, *7*, 313-339.

(55) Hamann, D.D. Structural failure in solid foods. In Physical Properties of Foods; Peleg, M., Bagley, E.B., Eds.; AVI Publishing Inc.: Connecticut, 1983; pp 305-340.

(56) Truong, V.D.; Daubert, C.R. Comparative study of large strain methods for assessing failure characteristics of selected food gels. *J. Texture Stud.* **2000**, *21*, 335-353.

(57) Foegeding, E.A. Molecular properties and functionality of proteins in food gels. In *Food Proteins*; Kinsella, J.E., Soucie, W.G., Eds.; AOCS Press: Phoenix, AZ, 1989; pp185-194.

(58) Puppo, M.C.; Añón, M.C. Effect of pH and protein concentration on rheological behavior of acidic soybean protein gels. *J. Agric. Food Chem*. **1998**, 46, 3039-3046.

(59) Wang, C.H.; Damodaran, S. Thermal gelation of globular proteins: influence of protein conformation on gel strength. *J. Agric. Food Chem*. **1991**, *39*, 433-438.

(60) Heertje, I.; van Kleef, F.S.M. Observations on the microstructure and rheology of ovalbumin gels. *Food Microstructure* **1986**, *5*, 91-98.

(61) Nir, I.; Feldman, Y.; Aserin, A.; Garti, N. Surface properties and emulsification behavior of denatured soy proteins. *J. Food Sci.* **1994**, *59*, 606-610.

MANUSCRIPT 1

Thermal Reversibility and Reformability of Commercial Soy Protein Isolate Gels

L.K. McKlem[a], T.C. Lanier[a], C.R. Daubert[a], and P. Kwanyuen[b]
[a]Department of Food Science, North Carolina State University, Raleigh, NC 27695-7624
[b]United States Department of Agriculture, Agricultural Research Service and Crop Science Department, North Carolina State University, Raleigh, NC 27695-7631

ABSTRACT

Gelling behavior of two commercially prepared soy protein isolates (SPI) was investigated during heating, cooling and reheating using both small strain rheology and vane fracture testing. These SPI, having been partially denatured during processing as evidenced by differential scanning calorimetry, gelled immediately upon hydration but were strengthened after heating (>50 ºC) and cooling. Holding at each endpoint temperature further increased final gel rigidity. When cooked gels were chopped they again reformed at 10 ºC and also strengthened upon subsequent heating and cooling. Additional thermal treatment did not enhance or diminish the gel properties. Results suggest that primarily noncovalent bonds (hydrogen bonds and hydrophobic interactions) and conformational changes during heating contribute to the structure of cooked gels made from these commercially processed SPI.

INTRODUCTION

Soy proteins are utilized extensively as ingredients in comminuted meat products for their functional contributions which include water holding, fat binding, and gelation. The ability of commercially prepared soy isolates to form gels below 80 °C is an important property because these meat products are seldom cooked in excess of this temperature (*1*).

Studies on soy protein gelation have largely focused on soy protein isolates (SPI) carefully produced in the laboratory and minimally denatured prior to characterization. In contrast, many commercially prepared SPI intended for use in comminuted meat products are intentionally denatured to some degree to improve their functionality (i.e. gelation). Such predenatured SPI behave quite differently from more native SPI, since their processing conditions have caused the proteins to assume various conformations and states of aggregation (*2*).

Since denaturation is a prerequisite for gel formation (*2*), isolates prepared in such a manner as to maintain the proteins in the near-native state typically gel only after heating. The two principal components of soy proteins, β-conglycinin and glycinin, exhibit different levels of heat stability which in turn influence their contribution to gel formation. When denatured, the less heat stable β-conglycinin has been observed to form weak gels, supported by noncovalent interactions, while after a higher temperature treatment, glycinin forms firm and elastic gels through both covalent and noncovalent interactions (*3-5*). Consequently, heating of these more native SPI to 80 ºC results in pastes which gel only upon cooling and which are thermally reversible (melt upon

reheating) (*6*). On the contrary, heating such SPI pastes to 100 ºC results in formation of a thermally irreversible gels (*7*).

In contrast to this behavior, commercially prepared SPI in which the proteins are more extensively denatured have been observed to gel immediately upon hydration (*8-10*). Commercially processed SPI can be expected to vary in gelation properties due to differences in processing conditions and varietal differences in soybean protein composition. For example, while Chronakis et al. (*8*) found that a commercial SPI formed gels that were less rigid after heating and cooling, Luck et al. (*10*), using a different commercially prepared SPI, found the opposite to be true.

Many researchers (*3, 4, 7, 11-13*) have concluded that a combination of molecular forces are involved in SPI gelation, including hydrogen bonding, hydrophobic interactions, and disulfide (covalent) bonding. Others (*6, 14, 15*) claim that soy protein gels are stabilized primarily by noncovalent bonds. This research was conducted to observe rheological changes in pastes prepared from commercial SPI, made from two soybean varieties, with regard to the thermal reversibility of any crosslinking (gel enhancing) reactions which might occur upon heating and cooling. Such information could help quantify the contribution of particular molecular forces responsible for gel formation.

MATERIALS AND METHODS

Preparation of Soy Protein Isolates

SPI were prepared from two soybean cultivars: *Prolina*, a high-protein variety developed by the USDA Agricultural Research Service and *Brim*, representative of most conventionally grown varieties. Soybeans were ground in a Retsch/Brinkman ZM1000 centrifugal grinding mill (Westbury, NY) equipped with 24-tooth rotor and 1.0 mm stainless steel ring sieve with motor speed set at 14000 rpm. Oil was extracted from the meal in a Soxhlet apparatus with warm hexane. The defatted meal was shipped to a processor, where SPI were produced by a proprietary commercial process to yield functional properties most useful in processed meat applications. Protein and moisture contents of the SPI as determined by the Dumus combustion method and the air oven method (*16*) respectively, were 87.1 % protein (w/w) and 6.4% moisture (w/w) for the *Prolina* isolate, and 85.4% protein (w/w) and 6.5% moisture (w/w) for the *Brim* isolate.

Differential Scanning Calorimetry (DSC)

A Perkin-Elmer DSC 7 (Norwalk, CT) differential scanning calorimeter using N_2 purge gas was used to assess the thermal denaturation of the commercially processed SPI. The DSC was calibrated with indium (enthalpy and onset temp) and dodecane (onset temp). SPI were hydrated (12% protein w/w) and approximately 65 mg was loaded into a stainless steel pan and hermetically sealed. A similarly weighted, empty stainless steel pan was used as the reference. The scanning temperature was raised from 5 to 140 ºC at a rate of 5 ºC/min. Temperatures at endothermic peak height (denaturation temperature,

T_d) and peak area (enthalpy) were recorded. Percent (%) native protein was calculated as area under peak for sample divided by area under peak for native SPI (as 100%) curve. Native SPI was prepared from the defatted *Prolina* and *Brim* by the method outlined by Lusas and Rhee (*17*).

Preparation of Gels for Small Strain Rheology

Sample pastes were prepared from *Prolina* and *Brim* SPI at 12% protein (w/w) in deionized water. Samples were vacuum packaged, mechanically blended for 5 min in a stomacher and stored at 5 ºC. The following day, samples were equilibrated to room temperature and macerated for an additional 5 min prior to small strain testing.

Small Strain Rheology during Heating/Cooling

Samples were analyzed in a StressTech Controlled Stress Rheometer (ATS Rheosystems Inc., Bordertown, NJ) using concentric cylinder geometry (CC25). G′ (storage modulus), a measure of gel rigidity, was continuously evaluated during various heating/holding/cooling cycles. Measurements were made within the linear viscoelastic region, determined previously for each sample at 10 ºC and 90 ºC. Samples were initially cooled from 25 ºC to a starting temperature of 10 ºC, then heated to endpoint temperatures ranging from 40 ºC to 90 ºC, held for either 0 or 30 min, and then cooled down to 10 ºC. All heating and cooling rates were 2.5 ºC/min. Samples were also coated with mineral oil to prevent moisture loss during heating. For selected trials, frequency sweeps (from 0.01 – 20 Hz at 10 Pa maximum stress) were performed initially at 25 ºC, after cooling to 10 ºC, after heating to the endpoint temperature, after holding for 30 min

at the endpoint temperature, and after cooling back to 10 ºC. At least three samples were tested for each heating/holding/cooling cycle, and G′ results are reported as averages of the data.

Vane Fracture Testing

Sample pastes were prepared from *Prolina* SPI at 12% protein (w/w) in deionized water. Samples were comminuted in a Stephan vertical cutter/mixer (Columbus, OH) at 80 bar vacuum, 900 rpm for 6 min at room temperature. Samples were placed into a plastic bag and vacuum-sealed to further reduce air pockets. Pastes were then extruded into 50 ml conical tubes (30 x 115 mm) using a manual sausage stuffer and tightly sealed to prevent moisture loss.

Samples were subsequently placed in a circulating water bath connected to a temperature controller. Samples were first equilibrated to 10 ºC, then heated to 80 ºC, held for 30 min, and then cooled to 10 ºC. All heating and cooling rates were 2.5 ºC/min, achieved through using additional external heating and cooling sources in combination with the circulating water bath and temperature controller. Samples were removed at 10 ºC (10), 80 ºC (80), 80 ºC after holding for 30 min (80/30), and at 10 ºC after cooling (10E) and vane fracture tests were immediately performed in triplicate at each temperature treatment. The vane (H = 20 mm, D = 10 mm) consisted of four identical stainless steel blades fixed to a center shaft. The vane was inserted flush with the sample surface and rotated with a Haake VT550 Viscometer (Gebruder Haake GmbH, Karlsruhe, Germany) at a speed of 0.5 rpm until fracture occurred. Peak torque (M_f) and time at

fracture (t_f) were recorded and stress at fracture (σ_f) and deformation at fracture (θ_f) were calculated according to Daubert et al. (*18*).

$$s_f = \frac{2M_f}{pD^3}\left(\frac{H}{D}+\frac{1}{6}\right)^{-1} \quad (1)$$

$$q_f = t_f\left(\frac{p}{60(s)}\right) \quad (2)$$

Statistical Analysis

All data were analyzed using the General Linear Models (GLM) procedure of SAS software (SAS Institute, Cary, NC). All statistical inferences reported were at least at a 5% probability level. Differences between treatment combinations were determined using paired comparisons (pdiff option) on the least square means at the original time intervals.

RESULTS AND DISCUSSION

DSC Measurements

DSC of *Prolina* and *Brim* SPI pastes revealed two endothermic peaks corresponding to denaturation of β-conglycinin (7S) and glycinin (11S). The denaturation temperatures (Td) for *Prolina* SPI were 76 and 95 ºC and Td for *Brim* SPI were 82 and 95 ºC **(Table 1)**. **Table 1** also reveals that *Prolina* and *Brim* SPI were partially denatured prior to heating.

Reported Td (peak temperature) of β-conglycinin range from 68-77 °C, while Td (peak temperature) of glycinin range from 85-92 °C (*2, 5, 8, 15, 19, 20*). Lakemond et al. (*21*) found that as the proportion of glycinin remaining native decreased from 95 to 5%, the Td of the glycinin remaining native increased from 88.5 to 95 °C. Elevated Td values for *Prolina* and *Brim* β-conglycinin and glycinin fractions may be due to partial denaturation from commercial processing as well as overall heterogeneity of soy proteins.

Small Strain Rheology

Frequency sweeps conducted prior to heating (at 25 °C) indicated that all *Prolina* and *Brim* SPI pastes had already formed gels. Other commercial SPI have previously been found to exhibit this property (*8-10*).

Effect of Heating Temperature and Holding Time on Rheology of Cooled Gels

When heated to 50 ºC or above and cooled to 10 ºC, SPI gels made from *Prolina* and *Brim* exhibited a rigidity (G′) higher than that obtained prior to heating (at 10 ºC) **(Figures 1** and **2)**. Furukawa et al. (*11*) subjectively classified 20% commercial SPI pastes heated to below 50 ºC as "soft" gels, while heating at temperatures from 60 ºC to 110 ºC induced "hard" gels, as determined using fracture testing. Gel hardness, measured at each elevated temperature, increased linearly with heating temperature up to 80 °C, which they attributed to increased hydrophobic interactions. By contrast, Chronakis (*9*) found that final G′ values (after heating from 30 ºC, holding for 30 min, and cooling to 5 °C) of commercially prepared SPI gels were greatest with a heating temperature of 60 °C and 40 °C, for 20% and 14% SPI gels respectively. Much lower final G′ values resulted

from higher heating temperatures. They attributed this to a conformational transition, caused by heating above 60 °C, from an aggregated to a more elastic, fine-stranded structure. We observed similar behavior in the gelation behavior of pastes made from a different commercially marketed SPI (data not shown).

The final rigidity (G′) of cooled *Prolina* and *Brim* gels increased with increasing endpoint temperature; addition of a holding time (30 min) at each endpoint temperature significantly increased gel rigidity ($p<0.05$) **(Figures 1** and **2)**. This would suggest the formation of thermally irreversible bonds (hydrophobic interactions and/or disulfide bonding) contribute to strengthening the gel network under these conditions (*5, 10, 11, 13*). Because heating to 80 ºC and holding for 30 min yielded such significant increases in final G′ values (and also exhibited the greatest increases during holding for 30 min), this treatment was selected for further testing.

Rheological Changes during Heating

Rigidity (G′) of all gels decreased as the temperature was increased **(Figure 3)**. Despite this decrease, all samples remained gelled (G′ > G″), as evidenced by frequency sweeps. This contrasts with the behavior during heating of SPI pastes made with less denatured protein; in such a case, increases in G′ near 70 °C and 90 °C are seen, corresponding to the denaturation temperatures of β-conglycinin and glycinin (*14, 15, 22*). The weakening of *Prolina* and *Brim* gels with increasing temperature is likely due to a reduction in hydrogen bonding, which would be largely reversible upon cooling (*5*). Any formation of (or strengthening of existing) hydrophobic and/or disulfide bonds during heating would oppose weakening of the gel that might otherwise result from

dissolution of hydrogen bonds. **Figure 3** demonstrates a diminishing slope as heating approaches the endpoint temperature. This could be attributed to formation of these gel-stabilizing bonds, or merely the completion of hydrogen bond dissolution.

Rheological Changes during Holding at the Endpoint Temperature

G′ of both *Prolina* and *Brim* gels increased during holding at 50 - 90 ºC, particularly between 60 and 80 ºC, likely indicating the formation of increased numbers of intermolecular hydrophobic interactions and/or disulfide bonds **(Figure 3)**. Similar results were found by Luck et al. (*10*) during holding of SPI gels at 90 °C. Any existing hydrophobic interactions stabilizing the gel would increase in strength with increasing temperature, to a maximum near 60 – 70 °C (*23*), and of course formation of new hydrophobic interactions would also strengthen the gel. An increase in the number of intermolecular disulfide bonds formed in the gel matrix would also be expected to enhance gel rigidity (*13, 24*). Renkema and van Vliet (*15*) similarly observed an increase in G′ during prolonged heating (0 – 5 h) of 12% native SPI at 90 ºC. They attributed this to increased incorporation of protein into the gel network (resulting from continued denaturation of proteins), as well as possibly to rearrangements occurring in the gel network. They also concluded that mainly noncovalent bonds were formed during gelation, although the exact nature of these bonds was not investigated.

Rheological Changes during Cooling

During cooling to10 ºC, G′ of all *Prolina* and *Brim* gels increased **(Figure 3)**. Gels heated to above 50 ºC increased in rigidity to significantly to higher G′ values than were measured before heating **(Figures 1** and **2)**. This would again suggest that

formation of thermally irreversible bonds (hydrophobic and/or covalent) occurred during these heating/holding treatments (*10*).

Prolina gels exhibited greater initial and final G′ values than *Brim* gels **(Figure 3)**. It may be that because *Prolina* SPI was more denatured by processing than the *Brim* SPI, and more reactive sites for bonding upon hydration and during subsequent heat treatment were readily available. *Prolina* proteins also possess more oxidizable free sulfhydryl groups in addition to an increased cysteine content, which could lead to increased disulfide bonding during heating that would strengthen the gel network (*25*). However, both *Prolina* and *Brim* gels, despite differing in initial (immediately upon hydration) gel rigidity, responded almost identically to heating and holding treatments ($p = 0.92$) **(Figures 1** and **2)**. It is also unlikely that intermolecular disulfide bonding contributes to the gel structure formed upon hydration of the *Prolina* SPI. Therefore, compositional differences between *Prolina* and *Brim* SPI may be an insignificant factor in explaining the overall higher rigidity of *Prolina* gels, but could influence the relative contribution of each type of intermolecular force contributing rigidity to the structure of cooked gels.

Variations in commercial processing conditions, even slight deviations, may have altered the resulting gel properties of the *Prolina* and *Brim* SPI. In previous research in our laboratory, *Prolina* and *Brim* isolates commercially processed in the same manner as SPI used in this study exhibited similar gelling behavior except that *Brim* gels displayed higher overall G′ values than *Prolina* gels (*10*). Several batches of commercially processed SPI would need to be evaluated to fully determine if differences in gel rigidity are due to processing effects or soybean composition.

Effect of Reheating

Gel rigidity (G′) after a second heating/holding/cooling cycle (to 80 ºC for 30 min) was equivalent to that induced by the initial heating/holding/cooling cycle **(Figure 4)**. Therefore, it is likely that changes in G′ noted during the initial cooling cycle and during the subsequent heating/holding/cooling cycle are all related to hydrogen bonding alone. Others have also found that a second heating/holding/cooling cycle had no effect on properties of gels formed initially by heating of less denatured soy proteins (*15, 26*). It is typical in globular protein gels for the modulus to increase substantially upon cooling and reversibly decrease upon reheating, provided the gels have been extensively cured (*27*).

Vane Fracture Testing

Upon heating to 80 ºC, *Prolina* gels exhibited a significant decrease ($p<0.05$) in fracture stress (gel strength) **(Figure 5A)**, which may be attributed to decreased hydrogen bonding. No change in deformability was evidenced **(Figure 6A)**. After holding at 80 ºC for 30 min, gel strength increased ($p<0.05$) **(Figure 5A)**, while gel deformability decreased ($p<0.05$) **(Figure 6A)**. Holding at 80 ºC thus induced gels to become stronger and more rigid, likely as a result of induced disulfide bonding and/or hydrophobic interactions between proteins. Increased disulfide bonding has been previously shown to decrease fracture strain (deformability) of soy protein gels (*28*). Upon cooling to 10 ºC, the gel network was strengthened, likely by increased hydrogen bonding **(Figure 5A)**. This gel strengthening was not accompanied by a decrease in gel deformability **(Figure 6A)**.

To determine if heated and cooled gels could be reformed after mechanical breakdown on the gel structure, the gels were again mechanically chopped and thermally processed by the same heating/holding/cooling regime. The gel formed by the rechopped material at 10 °C, prior to reheating, was stronger ($p<0.05$) and less deformable ($p<0.05$) **(10; Figures 5B** and **6B)** than the gel formed initially prior to any heating **(10; Figures 5A** and **6A)**. This rechopped gel and the initial gel were visually similar and could be classified as thick pastes. The rechopped gel again strengthened after heating and cooling and the strength ($p=0.96$) and deformability ($p=0.36$) after cooling was similar to the cooled gel resulting from the first heating/holding/cooling cycle **(10E; Figures 5B** and **6B)**. This gel was further chopped, reformed, and thermally processed twice more in an identical manner. Each time the reformed gel evidenced similar fracture stress and deformation at each temperature of the heating/holding/cooling cycle **(Figures 5** and **6)**.

These *Prolina* gels are obviously capable of reforming bonds after mechanical breakdown of the gel network and bonding forces are able to reform the gel network upon subsequent heating and cooling. However, it seems that protein-protein interactions develop during the first thermal treatment which lead to a slight degree of thermal irreversibility (or more stable conformation), since all reformed gels were stronger ($p<0.05$) and less deformable ($p<0.05$) **(10; Figures 5B-D** and **Figures 6B-D)** than the initial gel **(10; Figures 5A** and **6A)**.

Results from this investigation indicate that primarily noncovalent forces (hydrogen bonds and/or hydrophobic interactions) are involved in the gelation mechanism of these commercially prepared SPI. This is evidenced by the reformability of these gels as well as the similar effects of heating and holding on *Prolina* and *Brim*

gels. Differences in gel rigidity between *Prolina* and *Brim* gels are likely due to variations in primary processing rather than compositional differences since the gelation mechanisms are analogous.

LITERATURE CITED

(1) Foegeding, E.A.; Lanier, T.C. The contribution of nonmuscle proteins to texture of gelled muscle protein foods. In *Protein Quality and the Effects of Processing*; Phillips, R.D., Finley, J.W. Eds.; Marcel Dekker, Inc.: New York, 1989; 331-343.

(2) Hermansson, A.M. Soy protein gelation. *J. Amer. Oil Chem. Soc.* **1986,** *63,* 658-666.

(3) Utsumi, S.; Kinsella, J.E. Forces involved in soy protein gelation: effects of various reagents on the formation, hardness and solubility of heat-induced gels made from 7S, 11S, and soy isolate. *J. Food Sci.* **1985,** *50,* 1278-1282**.**

(4) Mori, T.; Nakamura, T.; Utsumi, S. Behavior of intermolecular bond formation in the late stage of heat-induced gelation of glycinin. *J. Agric. Food Chem.* **1986**, *34*, 33-36.

(5) Nagano, T.; Mori, H.; Nishinari, K. Effect of heating and cooling on the gelation kinetics of 7S globulin from soybeans. *J. Agric. Food Chem*. **1994**, *42*, 1415-1419.

(6) Catsimpoolas, N.; Meyer, E.W. Gelation phenomena of soybean globulins. I. Protein-protein interactions. *Cereal Chem.* **1970,** *47,* 559-570.

(7) Circle, S.J.; Meyer, E.W.; Whitney, R.W. Rheology of soy protein dispersions. Effect of heat and other factors on gelation. *Cereal Chem.* **1964,** *41,* 157-172.

(8) Chronakis, I.S.; Kasapis, S.; Richardson, R.K.; Doxastakis, G. Characterisation of a commercial soy isolate by physical techniques. *J. Texture Stud.* **1995,** *26,* 371-389.

(9) Chronakis, I.S. Network formation and viscoelastic properties of commercial soy protein dispersions: effect of heat treatment, pH and calcium ions. *Food Res. Int.* **1996,** *29(2),* 123-134.

(10) Luck, P.J.; Lanier, T.C.; Daubert, C.R.; Kwanyuen, P. Viscoelastic behavior of commercially processed soy isolate pastes during heating and cooling. *J. Food Sci.* **2002**, *67*, 1379-1382.

(11) Furukawa, T.; Ohta, S.; Yamamoto, A. Texture-structure relationships in heat-induced soy protein gels. *J. Texture Stud.* **1979,** *10,* 333-346.

(12) Nakamura, T.; Utsumi, S.; Mori, T. Interactions during heat-induced gelation in a mixed system of soybean 7S and 11S globulins. *Agric. Biol. Chem.* **1986**, *50*, 2429-2435.

(13) Shimada K.; Cheftel, J;C. Determination of sulfhydryl groups and disulfide bonds in heat-induced gels of soy protein isolate. *J. Agric. Food Chem.* **1988**, *36*, 147-153.

(14) Van Kleef, F.S.M. Thermally induced protein gelation: gelation and rheological characterization of highly concentrated ovalbumin and soybean protein gels. *Biopolymers* **1986**, *25*, 31-59.

(15) Renkema, J.M.S.; van Vliet, T. Heat-induced gel formation by soy proteins at neutral pH. *J. Agric. Food Chem.* **2002,** *50*, 1569-1573.

(16) AOAC. *Official Methods of Analysis*, 17th ed.; Association of Official Analytical Chemists: Arlington, VA, 2000.

(17) Lusas, E.W.; Rhee, K.C. Soy protein processing and utilization. In *Practical Handbook of Soybean Processing & Utilization*; Erickson, D.R., Ed.; AOCS Press: Champaign, IL, 1995; pp 138-139.

(18) Daubert, C.; Tkachuk, J.; Truong, V. Quantitative measurement of food spreadibility using the vane method. *J. Texture Stud.* **1998,** *29*, 427-435.

(19) Hermansson, A.-M. Physico-chemical aspects of soy protein structure formation. *J. Texture Stud.* **1978**, *9*, 33-58.

(20) Damodaran, S. Refolding of thermally unfolded soy proteins during the cooling regime of the gelation process: effect on gelation. *J. Agric. Food Chem.* **1988**, *36*, 262-269.

(21) Lakemond, C.M.M.; de Jongh, H.H.J.; Gruppen, H.; Voragen, A.G.J. Differences in denaturation of genetic variants of soy glycinin. *J. Agric. Food Chem*. **2002,** *50*, 4275-4281.

(22) Owen, S.R.; Tung, M.A.; Paulson, A.T. Thermorheological studies of food polymer dispersions. *J. Food Eng*. **1992**, *16*, 39-53.

(23) Damodaran, S. Amino acids, peptides, and proteins. In *Food Chemistry, 3rd ed*.; Fennema, O.R., Ed.; W.H. Freeman and Co.: New York, 1996; 321-430.

(24) Furukawa, T.; Ohta, S. Mechanical and water-holding properties of heat-induced soy protein gels as related to their structural aspects. *J. Texture Stud.* **1982**, *13*, 59-69.

(25) Kwanyuen, P.; Wilson, R.F.; Burton, J.W. Soybean Protein Quality. In *Emerging Technologies, Current Practices, Quality Control, Technology Transfer, and Environmental Issues*; Koseoglu, S.S., Rhee K.C., Wilson, R.F., Eds.; Volume I in The Proceedings of the World Conference on Oilseed and Edible Oils Processing: Champaign, IL, 1998; pp 284-289.

(26) Renkema, J.M.S.; Knabben, J.H.M.; van Vliet, T. Gel formation by â-conglycinin and glycinin and their mixtures. *Food Hydrocolloids* **2001,** *15,* 407-414.

(27) Clark, A.H. Gelation of globular proteins. In *Functional Properties of Food Macromolecules*; Hill, S.E., Ledward, D.A., Mitchell, J.R., Eds.; Chapman & Hall: Gaithersburg, Maryland, 1998; pp77-143.

(28) Puppo, M.C.; Añón, M.C. Effect of pH and protein concentration on rheological behavior of acidic soybean protein gels. *J. Agric. Food Chem*. **1998**, 46, 3039-3046.

Table 1. Percent (%) Native Protein and Denaturation Temperature (Td) for *Prolina* and *Brim* SPI (12% protein w/w) as Determined by DSC

Sample	% Native Protein	Td (ºC)	
		β-conglycinin	Glycinin
Prolina	21	76	95
Brim	39	82	95

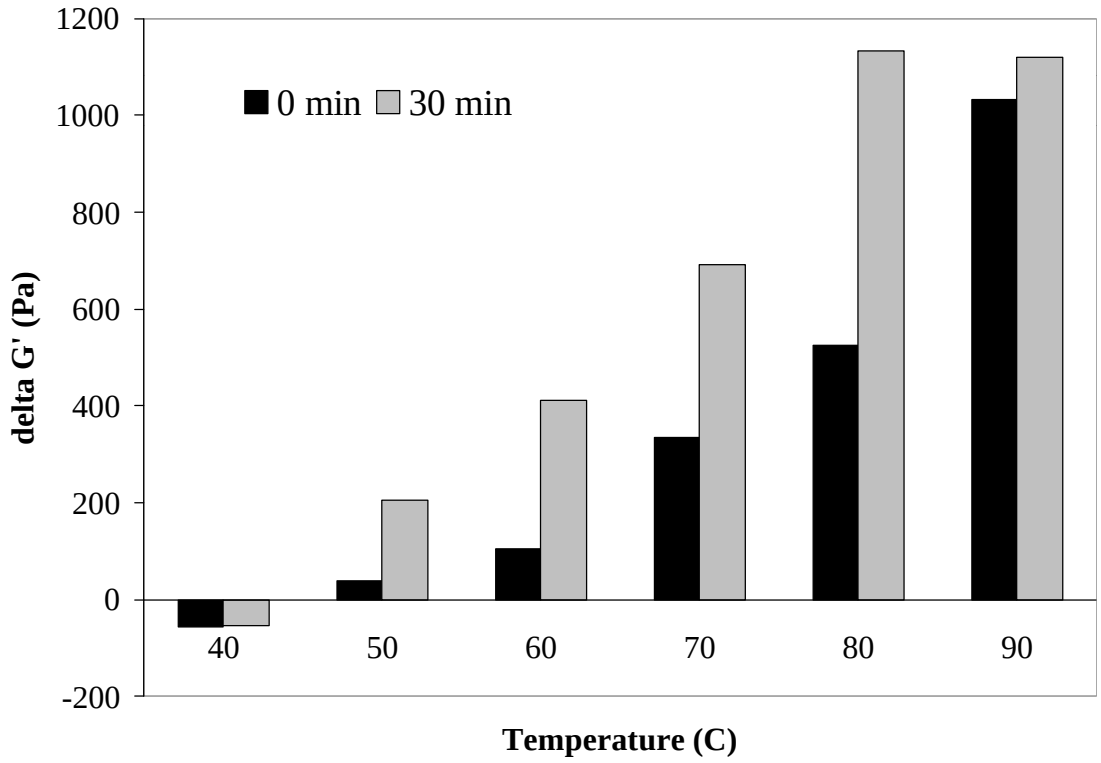


Figure 1. Effect of temperature and holding time on rigidity of *Prolina* gels (12% protein w/w). Delta G′ values represent the final G′ values (after heating, holding, and cooling) minus the initial G′ values (at 10 ºC).

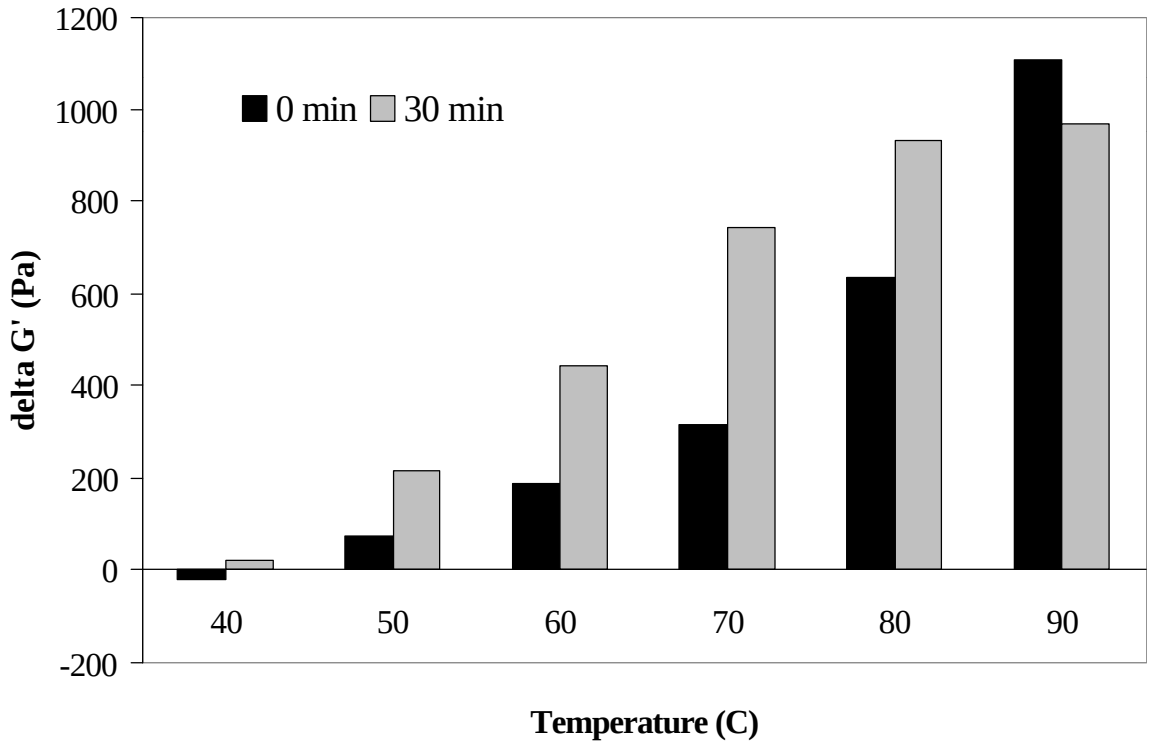


Figure 2. Effect of temperature and holding time on rigidity of *Brim* gels (12% protein w/w). Delta G′ values represent the final G′ values (after heating, holding, and cooling) minus the initial G′ values (at 10 ºC).

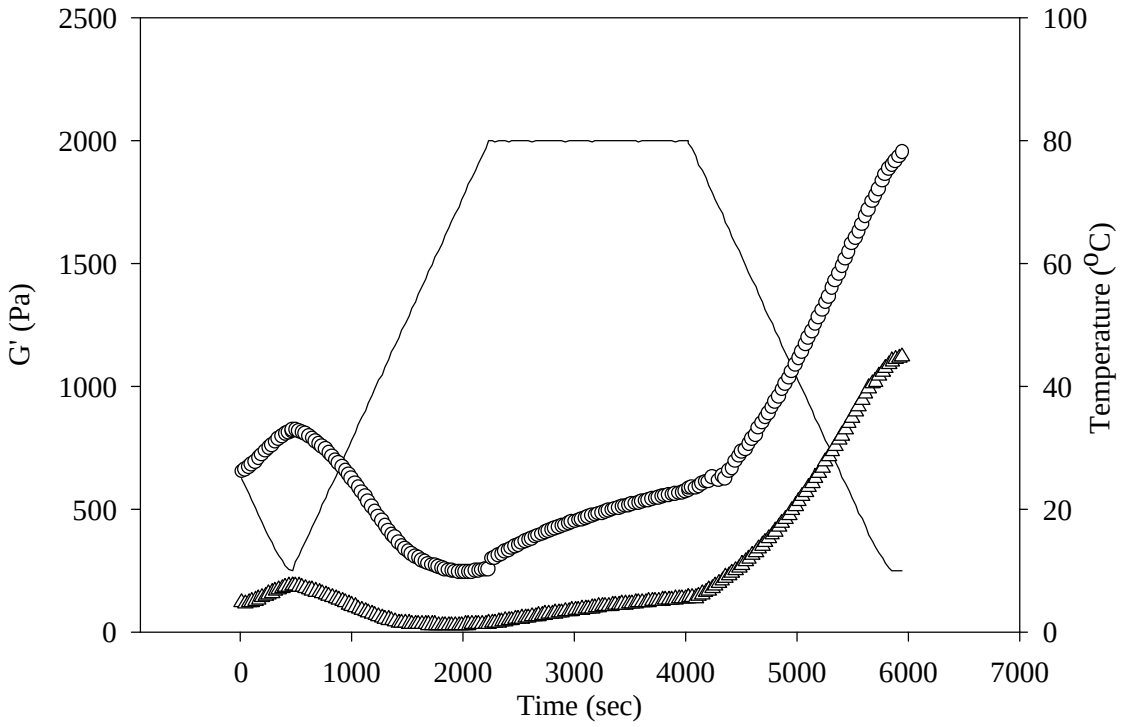


Figure 3. Monitoring of G′ during heating to 80 ºC, holding at 80 ºC for 30 min, and cooling to 10 ºC of *Prolina* and *Brim* gels (12% protein w/w). O *Prolina*, Δ *Brim*, – Temperature.

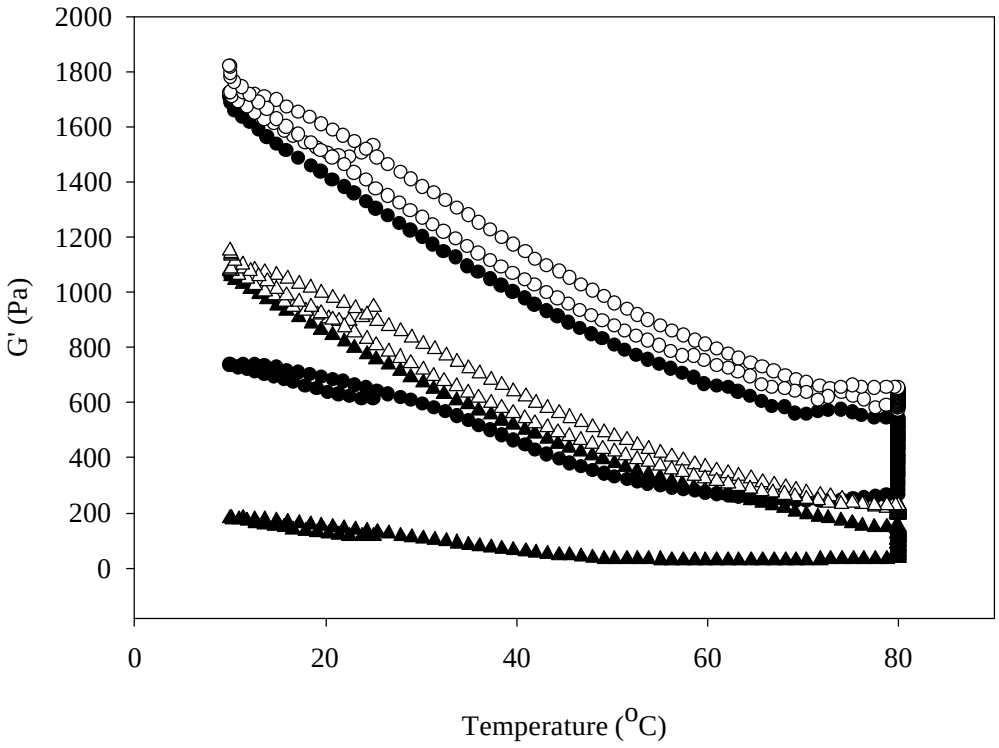


Figure 4. Monitoring of G′ during duplicate heating, holding, and cooling cycles of *Prolina* and *Brim* gels (12% protein w/w).● *Prolina*, O *Prolina* (reheating), ▲*Brim*, ∆ *Brim* (reheating).

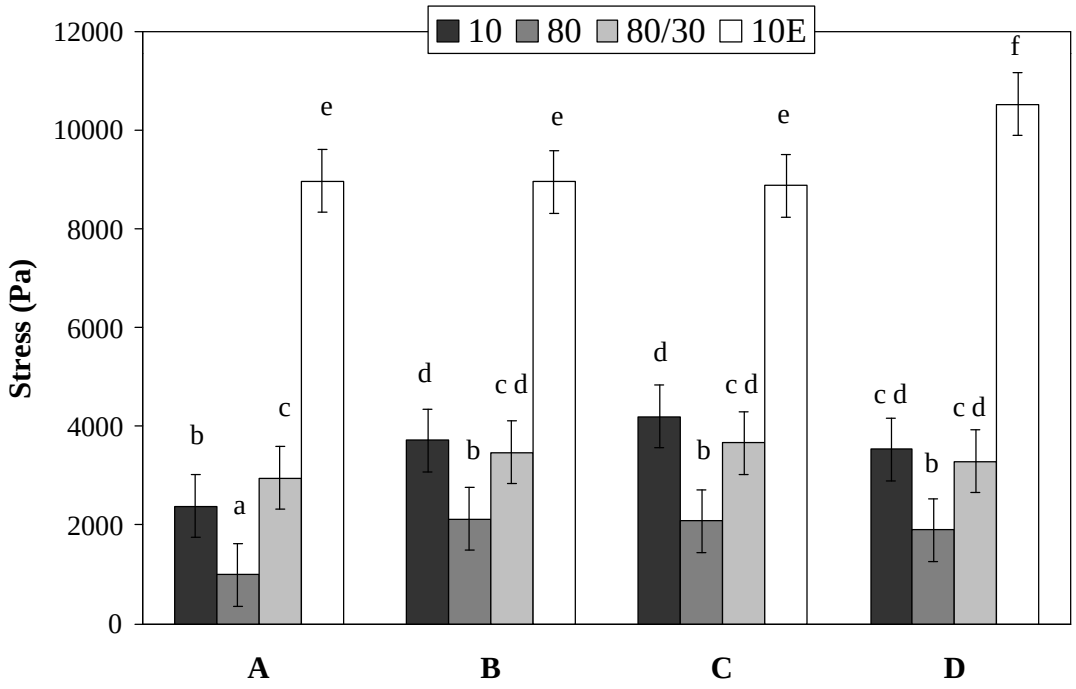


Figure 5. Stress at fracture of *Prolina* gels as determined by vane fracture testing during each stage (10, 80, 80/30, 10E) of four heating, holding, and cooling cycles (A-D). Error bars are standard errors from the analysis of variance. Bars with different letters are significantly different ($p<0.05$) from each other.

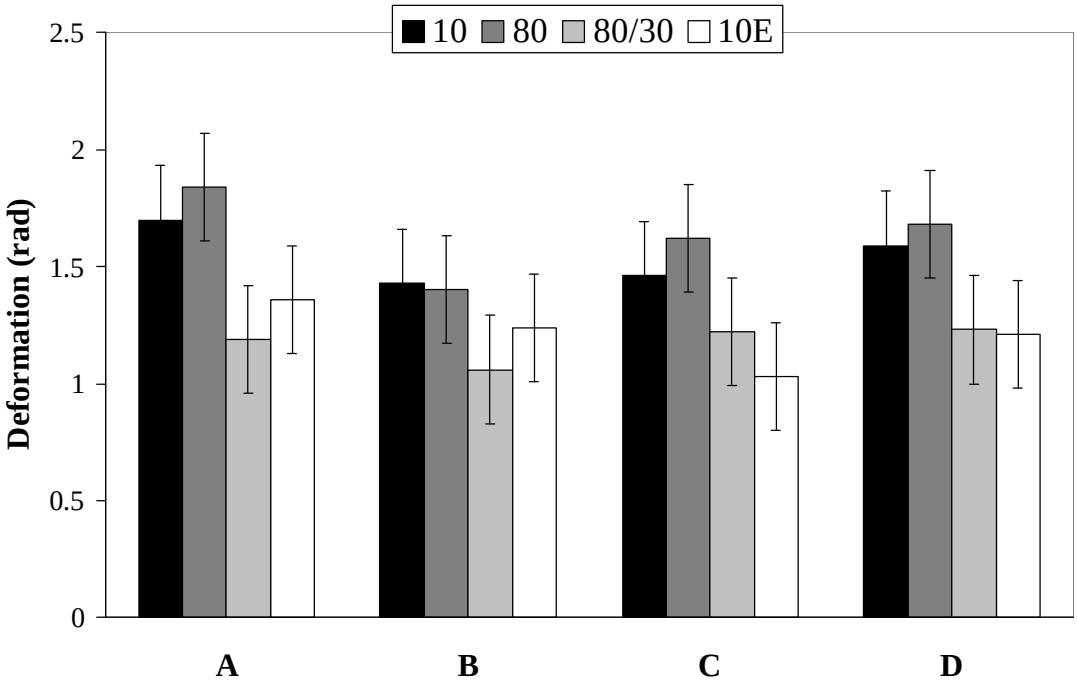


Figure 6. Deformation at fracture of *Prolina* gels as determined by vane fracture testing during each stage (10, 80, 80/30, 10E) of four heating, holding, and cooling cycles (A-D). Error bars are standard errors from the analysis of variance.

MANUSCRIPT 2

Molecular Forces Contributing to Gelation of Commercial Soy Protein Isolates

L.K. McKlem[a], T.C. Lanier[a], and P. Kwanyuen[b]
[a]Department of Food Science, North Carolina State University, Raleigh, NC 27695-7624
[b]United States Department of Agriculture, Agricultural Research Service and Crop Science Department, North Carolina State University, Raleigh, NC 27695-7631

ABSTRACT

Chemical reagents were added to commercial soy protein isolate (SPI) gels to determine contribution of covalent (disulfide bonding) and noncovalent (hydrogen bonding and hydrophobic interactions) forces during different stages of the gelation process. Small strain rheology and gel solubilization were utilized to study the effects of urea and/or dithiothreitol (DTT) addition. These treatments decreased final G′ values of SPI gels, but neither prevented gel formation. Gels were completely solubilized in solution containing urea and DTT. A high degree of solubilization in DTT, and formation of gels prior to heating by pastes containing urea, indicated that disulfide bonding was important in the initial gel formation of these predenatured SPI. Increases in G′ during holding at 80 ºC and upon cooling were attributed to hydrophobic interactions and hydrogen bonding, respectively. Some differences in gelling response due to soybean type, and therefore protein composition, were noted.

INTRODUCTION

Soy proteins are mainly comprised of two storage proteins, β-conglycinin (7S) and glycinin (11S). The ratio of β-conglycinin to glycinin varies between soybean varieties and influences formation and properties of soy protein isolate (SPI) gels (*1, 2*). Gels made from β-conglycinin alone are weaker than glycinin gels and are thermally reversible, indicating they are stabilized primarily by hydrogen bonding and hydrophobic interactions (*1, 3, 4*). Disulfide bonding in β-conglycinin has not been demonstrated, presumably due to the low content of cysteine in this fraction of most conventionally grown soybean varieties (*5*). Conversely, glycinin is sulfur-rich (*5*) and forms heat-induced gels which exhibit thermal irreversibility (*6*) and a more firm and elastic texture than β-conglycinin gels (*2, 6*). Disulfide bonding, hydrophobic interactions, and hydrogen bonds all seem to contribute to formation of soy glycinin gel networks (*1, 3, 6*).

Denaturants such as urea and guanidinium hydrochloride (GuHCl) affect the structure of water and can be utilized to weaken hydrophobic interactions and hydrogen bonds between soy proteins (*3, 4, 7, 8*). The importance of disulfide bonding in SPI gels has been demonstrated by addition of sulfhydryl blocking agents, N-ethylmaleimide (NEM) or disulfide reducing agents dithiothreitol (DTT) or 2-mercaptoethanol (ME) (*3, 7, 9, 10*). Although disulfide bonds are not essential for SPI gelation, they are crucial in forming a strong, elastic gel (*3, 10*).

Effects of such reagents on gel formation and rheology (*3, 4, 7, 10*) or on solubilization of gels already formed (*3, 7, 9, 10*) have been typical means used to probe the bonding forces responsible for protein gel formation. The present goal was to apply these methods to determine relative contribution of hydrogen bonding, hydrophobic

interactions, and disulfide bonding in the gelation of SPI prepared by a commercial process from two different soybean cultivars.

MATERIALS AND METHODS

Preparation of Soy Protein Isolates

SPI were prepared from two soybean cultivars: *Prolina*, a high-protein variety developed by the USDA Agricultural Research Service and *Brim*, representative of most conventionally grown varieties by the method previously outlined (*11*).

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Proteins from *Prolina* and *Brim* SPI were dissociated and separated using a Bio-Rad (Richmond, CA) Protean II slab gel apparatus according to Chua (*12*) with the following modifications. *Prolina* and *Brim* isolates were dissociated in 50 mM Tris-HCl, pH 8.0, 2% (w/v) SDS, and 0.1 M β-mercaptoethanol in boiling water bath for 10 min. Samples containing approximately 100 μg protein were loaded onto SDS-PAGE gel and proteins were separated using a linear 10-20% gradient polyacrylamide gel. Electrophoresis was carried out at 10 mA/1.5 mm-thick gel until tracking dye reached the bottom of the gel. Gels were stained with Coomassie brilliant blue R-250, then destained and dried with a Bio-Rad GelAir Dryer (Richmond, CA).

Dried gels were scanned with a Molecular Dynamics Personal Densitometer SI (Sunnyvale, CA) equipped with a HeNe laser light source. ImageQuant software for volume integration was used to analyze data. Total optical density (OD) was determined

for entire protein bands. Apparent OD of each protein was obtained by subtracting the local average background OD from the total OD of protein bands within the same gel volume.

Preparation of Gels and Small Strain Rheology

Sample pastes were prepared at 12% protein (w/w) in deionized water (control), 8 M urea, 10 mM DTT, or 8 M urea + 10 mM DTT. Samples were vacuum packaged, mechanically macerated for 5 min in a stomacher and stored at 5 ºC. The following day, samples were equilibrated to room temperature and macerated for an additional 5 min. Chemical modifiers, urea and DTT, were purchased from Sigma Chemical Co. and all reagents were prepared fresh and used within two days.

Samples were analyzed during heating and cooling on a StressTech Controlled Stress Rheometer (ATS Rheosystems Inc., Bordertown, NJ) using concentric cylinder geometry (CC25) by the same method previously described (*11*).

Gel Solubilization by Chemical Reagents

Prolina and *Brim* gels (12% protein w/w) were partitioned into two groups: unheated and heated. For the heated samples, gels were heated from 10 ºC to 80 ºC at 2.5 ºC/min, held for 30 min at 80 ºC, and cooled to 10 ºC at 2.5 ºC/min in a circulating water bath connected to a temperature controller. All gels (unheated and heated) were subsequently mixed (2 g in 10 ml) with the following solutions: deionized water (control), 8 M urea, 10 mM DTT, or 8 M urea + 10 mM DTT, then incubated at 25 ºC for 18 hours with agitation. After incubation, samples were centrifuged at 8000 rpm for 10

min and the protein in the supernatant was quantified by the Biuret method. The percent (%) solubility of the protein was determined as follows

$$\% \text{ solubility} = \frac{\text{concentration (mg/ml) protein in the supernatant}}{120 \text{ mg/ml}} \times 100 \qquad (1)$$

Statistical Analysis

Gel solubilization data were analyzed using the General Linear Models (GLM) procedure of SAS software (SAS Institute, Cary, NC). All statistical inferences reported were at least at a 5% probability level. Differences between treatment combinations were determined using paired comparisons (pdiff option) on the least square means at the original time intervals.

RESULTS AND DISCUSSION

SDS-PAGE

Prolina, the higher-protein cultivar, exhibited a higher percentage of total storage protein than *Brim* and also contained about 10% greater β-conglycinin with a similar content of glycinin **(Table 1)**. *Prolina* was previously shown to contain 5 times more cysteine residues per mole protein in the β-conglycinin fraction than conventional cultivars like *Brim* (*13*).

Small Strain Rheology

Frequency sweeps conducted prior to heating indicated that *Prolina* and *Brim* SPI

pastes prepared in deionized water or urea had formed gels, whereas samples containing DTT had not **(Figures 1** and **2)**. *Prolina* gels prepared in urea had slightly higher G′ values than the control gels, whereas *Brim* gels prepared in either urea or deionized water had similar G′ values.

Addition of 8 M urea may not be sufficient to dissipate all hydrogen bonding and hydrophobic interactions in the 12% soy protein gels as formation of gels in urea can occur in concentrated protein solutions (*14*). Disulfide bonds are likely important in the initial gel structure of both of these isolates, since DTT addition completely disintegrated the gel network. It is unlikely though, that intermolecular disulfide bonds would form upon hydration. Therefore, intramolecular disulfide bonds between acidic and basic subunits of glycinin were likely reduced by DTT addition (*15*) and contribute to stabilization of the gel network.

Samples that gelled initially exhibited a decrease in G′ as the temperature was increased to 80 °C **(Figures 1** and **2)**, which could be attributed to decreased (but thermally reversible) hydrogen bonding. G′ increased significantly during holding at 80°C for the control *Prolina* and *Brim* gels. An increase in G′ during holding was also noted for gels prepared in DTT. It therefore seems likely that hydrophobic interactions were responsible for strengthening the gel network during the 30-min holding period, since higher temperatures weaken hydrogen bonding but strengthen hydrophobic interactions (*7*, *8*). These temperatures should also favor oxidation of cysteine and sulfhydryl-disulfide interchange (*10*), but increases in rigidity during holding are not likely due to disulfide bonding since an increase in gel rigidity was not evident in urea-added samples.

During subsequent cooling to 10 °C, all *Prolina* and *Brim* gels (except urea + DTT pastes) exhibited an increase in G′ **(Figures 1** and **2)**. Control gels and those made with DTT increased to G′ values greater than those measured prior to heat treatment (at 10 °C), whereas gels made with urea did not exhibit such thermal irreversibility. Frequency sweeps confirmed that the DTT-added sample had now gelled (G′ > G″), as were the control and urea treatments.

Urea addition reduced G′ of *Brim* gels more than *Prolina*, whereas DTT addition had a similar effect on the two SPI **(Figures 1** and **2)**. *Prolina* gels also exhibited overall higher G′ values than *Brim* gels for the control and urea samples, which may imply increased importance of disulfide bonding to *Prolina* gels. When final (heated and cooled) G′ values of the urea- and DTT-added samples were added, the sum approximated the final G′ value of the *Prolina* or *Brim* gels prepared with no chemical reagents **(Figure 3)**.

Thus it would appear that the contribution of disulfide bonding to this final (cooled) gel rigidity could be calculated as G′ of the urea-added sample divided by G′ (final) of the control; likewise, the contribution of hydrogen + hydrophobic bonding would correspond to G′ (final) of the DTT-added sample divided by G′ (final) of the control. Such an analysis again suggests that disulfide bonding (mainly intramolecular) is a more important contributor to the rigidity of *Prolina* gels following heating and cooling. Recall that *Prolina* has an increased number of cysteine residues (especially in the 7S fraction which is present at a higher ratio to the 11S fraction than in *Brim*) which could participate in disulfide bonding reactions. Conversely, hydrogen bonding and/or

hydrophobic interactions seem more important to gelation of *Brim* isolates after heating and cooling. The relative contribution of forces stabilizing the gel may thus depend upon soybean cultivar.

Note that addition of urea + DTT did not result in gel formation by either *Prolina* or *Brim* SPI **(Figures 1** and **2)**. This further indicates necessity of both covalent and noncovalent bonding forces in formation of the gel network.

Gel Solubilization by Chemical Reagents

In control samples, more protein was solubilized by deionized water from heated and unheated *Brim* gels ($p<0.05$) **(Table 2)**. In the present work, *Brim* gels also exhibited lower gel rigidity values than *Prolina* gels **(Figures 1** and **2)**. Heating significantly decreased gel solubility of both *Prolina* and *Brim* control samples ($p<0.05$) **(Table 2)** and heated and cooled gels were correspondingly more rigid **(Figures 1** and **2)**. An inverse correlation between gel solubility and gel hardness has been noted by others (*3*, *7*, *9*).

About 90% protein was solubilized from heated and unheated *Prolina* and *Brim* gels due to the addition of urea **(Table 2)** indicating the importance of hydrogen bonding and hydrophobic interactions to the gel network. Shimada and Cheftel (*10*) found that protein solubility in 6 M urea + 0.5% SDS was 97% for unheated SPI and about 90% for heated SPI. Protein solubility was near 100% in buffer containing 6 M urea, 0.5% SDS, and 10 mM DTT. They concluded that about 10% of soy proteins are insolubilized due to the formation of intermolecular disulfide bonds during heating. Since protein solubilization of *Prolina* and *Brim* gels in urea did not decrease due to heat treatment, it seems likely that intermolecular disulfide bonding was minimal during heating and

holding. The 10% insolubilization therefore is likely due to the presence of intramolecular disulfide bonds between soy protein subunits.

Addition of DTT also increased the solubility of heated and unheated *Prolina* and *Brim* gels **(Table 2)**. Other researchers found that addition of 0.2 M ME increased solubility of 12% SPI gels from 50% to 90% (*3, 9*). Less protein was solubilized from the heated gels than unheated gels ($p<0.05$). This data suggests that additional noncovalent interactions were formed during heating, holding, and cooling. Previous work has demonstrated that primarily noncovalent interactions are formed during heating and cooling of *Prolina* and *Brim* gels (*11*).

Results from this investigation indicate that hydrogen bonding, hydrophobic interactions, and disulfide bonding all play roles in commercial SPI gelation. Disulfide bonding and hydrogen bonding are the major forces involved in gelation of SPI before heating, while hydrophobic interactions participate in the gel network as well after heating and cooling. Intramolecular disulfide bonds are important in maintaining the subunit structure of glycinin and are likely the main form of disulfide bonds involved in adding rigidity to the gel network. However, they are not essential for gelation, since reduction by DTT still produced gels after heating and cooling of both *Prolina* and *Brim* SPI. The greater contribution of disulfide bonding evidenced in *Prolina* gels is likely due to its increased cysteine content. Results from gel solubilization experiments and previous work (*11*) indicate that intermolecular disulfide bond formation during heating, holding, and cooling is minimal. Therefore, increasing gel rigidity of *Prolina* and *Brim* gels during holding and cooling is likely due to hydrophobic interactions and hydrogen bonding, respectively.

LITERATURE CITED

(1) Nakamura, T.; Utsumi, S.; Mori, T. Interactions during heat-induced gelation in a mixed system of soybean 7S and 11S globulins. *Agric. Biol. Chem.* **1986,** *50*, 2429-2435.

(2) Renkema, J.M.S.; Knabben, J.H.M.; van Vliet, T. Gel formation by â-conglycinin and glycinin and their mixtures. *Food Hydrocolloids* **2001,** *15*, 407-414.

(3) Utsumi, S.; Kinsella, J.E. Forces involved in soy protein gelation: effects of various reagents on the formation, hardness and solubility of heat-induced gels made from 7S, 11S, and soy isolate. *J. Food Sci.* **1985,** *50*, 1278-1282.

(4) Nagano, T.; Mori, H.; Nishinari, K. Effect of heating and cooling on the gelation kinetics of 7S globulin from soybeans. *J. Agric. Food Chem.* **1994,** *42*, 1415-1419.

(5) Kinsella, J.E. Functional properties of soy proteins. *J. Amer. Oil Chem. Soc.* **1979,** *56*, 242-258.

(6) Mori, T.; Nakamura, T.; Utsumi, S. Behavior of intermolecular bond formation in the late stage of heat-induced gelation of glycinin. *J. Agric. Food Chem.* **1986,** *34*, 33-36.

(7) Furukawa, T.; Ohta, S.; Yamamoto, A. Texture-structure relationships in heat-induced soy protein gels. *J. Texture Stud.* **1979,** *10*, 333-346.

(8) Damodaran, S. Amino acids, peptides, and proteins. In *Food Chemistry, 3rd ed.*; Fennema, O.R., Ed.; W.H. Freeman and Co.: New York, 1996; 321-430.

(9) Ustumi, S.; Kinsella, J.E. Structure-function relationships in food proteins: subunit interactions in heat-induced gelation of 7S, 11S, and soy isolate proteins. *J. Agric. Food Chem.* **1985,** *33*, 297-303.

(10) Shimada, K.; Cheftel, J.C. Determination of sulfhydryl groups and disulfide bonds in heat-induced gels of soy protein isolate. *J. Agric. Food Chem.* **1988,** *36*, 147-153.

(11) McKlem, L.K.; Lanier, T.C.; Kwanyuen, P. Thermal reversibility and reformability of commercial soy protein isolate gels. To be submitted to *J. Agric. Food Chem.* **2002**.

(12) Chua, N.H. Electrophorectic analysis of chloroplast proteins. *Meths. Enzymol.* **1980,** *69*, 434-446.

(13) Kwanyuen, P.; Wilson, R.F.; Burton, J.W. Soybean Protein Quality. In *Emerging Technologies, Current Practices, Quality Control, Technology Transfer, and Environmental Issues*; Koseoglu, S.S., Rhee K.C., Wilson, R.F., Eds.; Volume I in The Proceedings of the World Conference on Oilseed and Edible Oils Processing: Champaign, IL, 1998; pp 284-289.

(14) Huggins, C.; Tapley, D.F.; Jensen, E.V. Sulphydryl-disulphide relationships in the induction of gels in proteins by urea. *Nature* **1951,** *167*, 592-593.

(15) Kim, S.H.; Kinsella, J.E. Effects of reduction with dithiothreitol on some molecular properties of soy glycinin. *J. Agric. Food Chem.* **1986,** *34*, 623-627.

Table 1. Storage Protein Composition of *Prolina* and *Brim* SPI

Isolate	% β-conglycinin (7S)	% Glycinin (11S)	11S+7S
Prolina	31.2	46.4	77.6
Brim	21.8	51.5	73.3

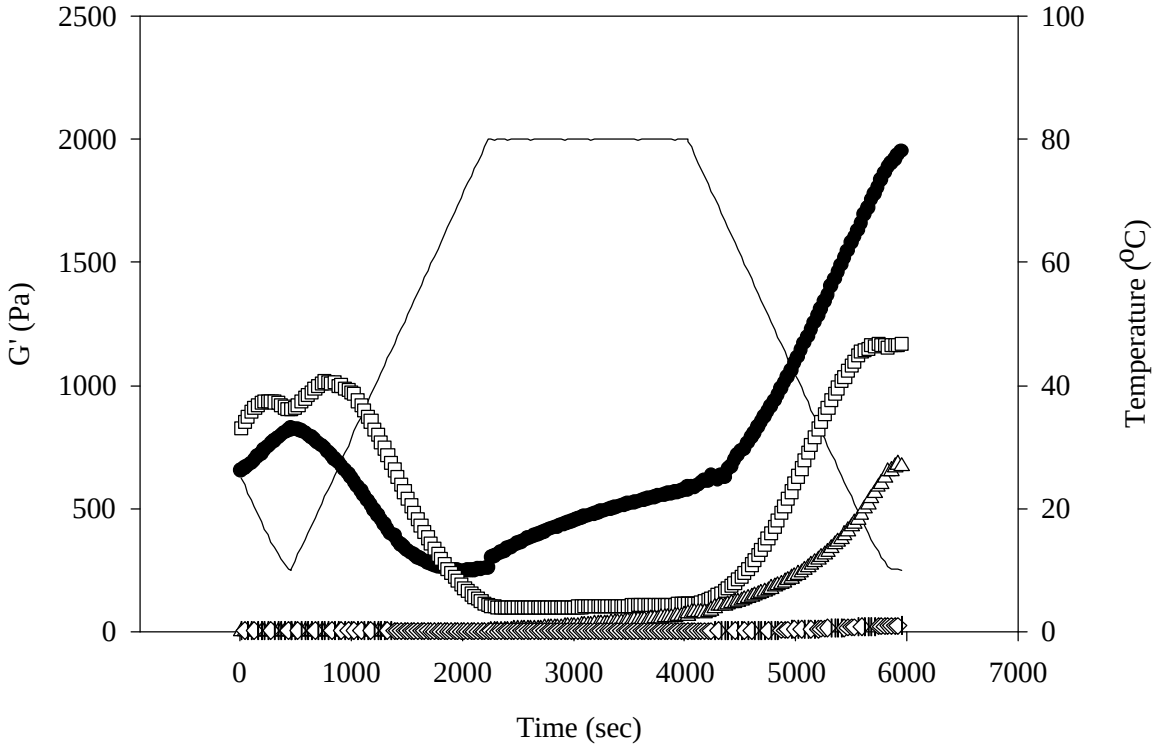


Figure 1. Effect of 8 M urea, 10 mM DTT, and 8 M urea + 10 mM DTT on *Prolina* SPI pastes (12% protein w/w) during heating and cooling. ● Control, □ 8 M urea, Δ 10 mM DTT, ◊ 8 M urea + 10 mM DTT, – Temperature.

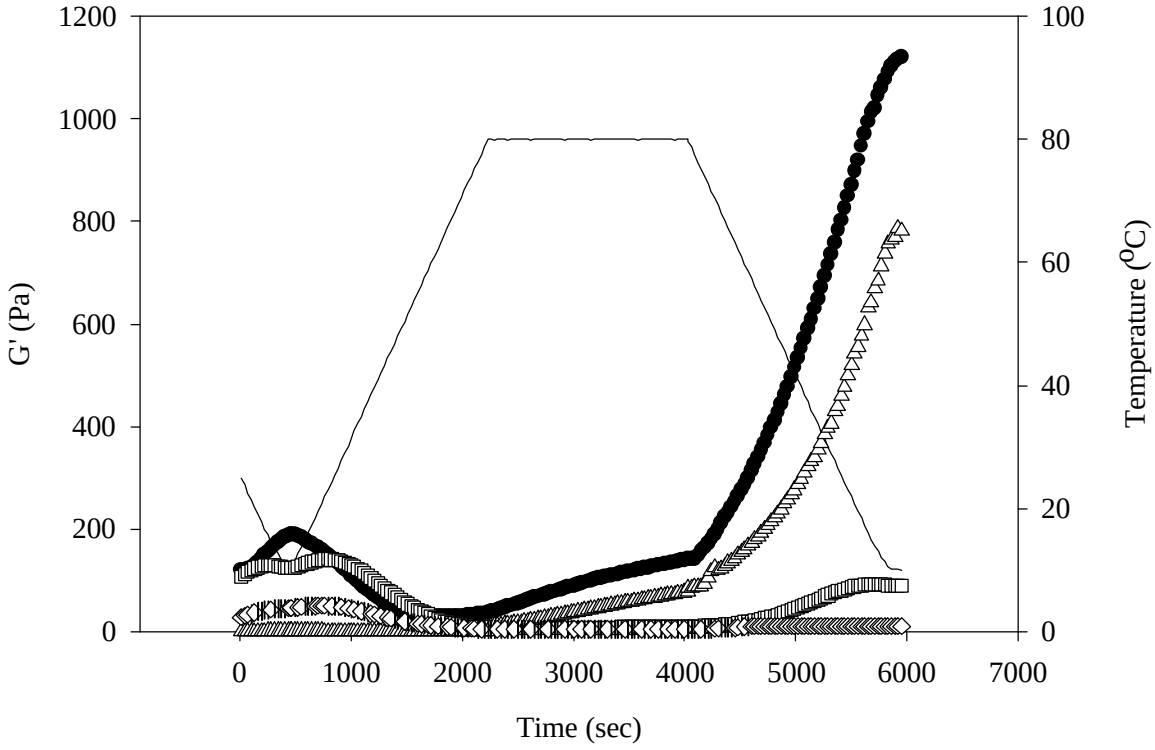


Figure 2. Effect of 8 M urea, 10 mM DTT, and 8 M urea + 10 mM DTT on *Brim* SPI pastes (12% protein w/w) during heating and cooling.● Control,□ 8 M urea, Δ 10 mM DTT, ◊ 8 M urea + 10 mM DTT, – Temperature.

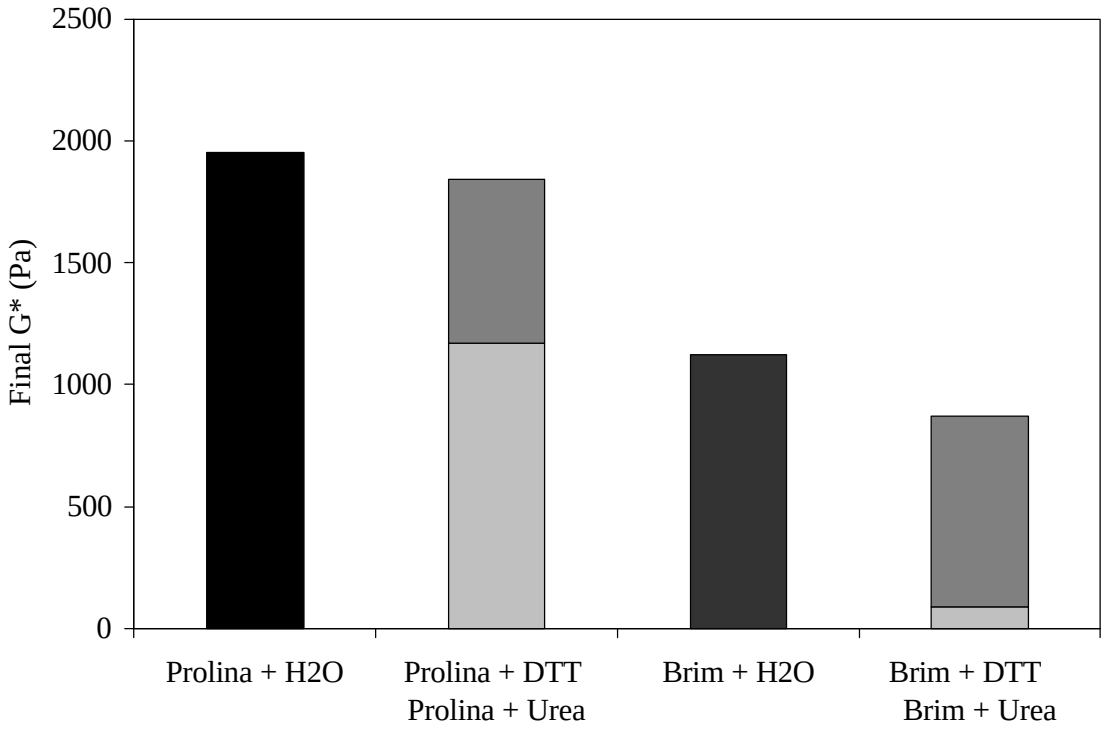


Figure 3. Representation of the molecular forces involved in *Prolina* and *Brim* SPI gelation. ■ H2O, ▣ + DTT, □ + Urea.

Table 2. Effect of Various Chemical Reagents on the Percent Solubilization of Proteins from Gels made from *Prolina* and *Brim* SPI

	Prolina		*Brim*	
Treatment	Unheated	Heated	Unheated	Heated
Control	48 c	24 a	72 d	37 b
8 M Urea	84 e	90 f	89 ef	91 f
10 mM DTT	91 fg	68 d	94 gh	90 f
8 M Urea + 10 mM DTT	97 ij	100 ijk	101 k	97 hi

Values with different letters are significantly different from each other ($p<0.05$)