ABSTRACT

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Of Particulate Whey Protein Isolate Gels. (Under the direction of E. Allen Foegeding)

Whey protein isolate (WPI) forms particulate, heat-induced gels under conditions of low electrostatic repulsion among proteins. Particulate gels appear opaque, release water when deformed, and can be formed by 1) adjusting the pH to near the isoelectric point (pI) of β -lactoglobulin ("pH treatment"), and 2) addition of >200 mM NaCl at pH 7.0 ("NaCl treatment"). The objective of our research was to understand the electrostatic contribution to particulate gel formation, using large strain rheology and microstructural techniques to characterize gel properties.

Gels of WPI (10% protein w/v) were formed by heating at 80°C for 30 minutes. The large strain fracture properties of shear strain at fracture (γ_f), shear stress at fracture (σ_f), and slope ratio ($R_{0.3}$) (indication of non-linear stress/strain behavior) were measured with a torsion gelometer. Gel microstructure was observed using scanning electron microscopy (SEM). Gel permeability (B_{gel}) was evaluated by measuring the flow of solvent through a fixed gel matrix and calculating the permeability coefficient based on Darcy's Law.

Fracture properties for both treatments were determined from pH 5.2 to 5.8 and NaCl concentration from 0.2 to 0.6 M (pH 7). When σ_f and γ_f curves for pH and NaCl treatments were overlaid, there was a distinct overlap. Based on this, treatment pairs were formulated to match rheological properties. The "high stress" pair was matched at pH 5.47 and 0.25 M NaCl, pH 7.0 ($\sigma_f \approx 23$ kPa and $\gamma_f \approx 1.86$) and the "low stress" pair at pH 5.68 and 0.6 M NaCl, pH 7.0 ($\sigma_f \approx 13$ kPa and $\gamma_f \approx 1.90$). Each pair was not significantly different for σ_f ($p > 0.05$), and

there was no significant difference among all treatment pair values for γ_f ($p>0.05$). However, the $R_{0.3}$ was treatment, not pair specific. The NaCl treatments (0.25 M NaCl, pH 7 and 0.6 M, pH 7) had $R_{0.3}$ values of 1.23 and 1.26, respectively, which were not significantly different ($p>0.05$). The gels formed at pH 5.47 and 5.68 had $R_{0.3}$ values of 1.05 and 1.11, respectively, which were significantly different ($p<0.05$). Moreover, $R_{0.3}$ shifted upward to 1.29 as the pH is increased to 5.8. Interestingly, when the sulfhydryl blocker n-ethylmaleimide was added at 2 mM to the 0.25 M NaCl, pH 7 gel treatment, its rheological behavior was NSD ($p>0.05$) to the pH 5.47 gel treatment (i.e. the $R_{0.3}$ fell to 1.06). Increasing values of strain hardening ($R_{0.3} > 1$) have been related to the presence of more than one structural component in the gel network (Bot et al., 1996). The increased strain hardening for NaCl treatments may be largely due to intermolecular disulfide bond formation, which is preferred at pH 7 relative to isoelectric pH due to the pKa of the thiol group. Random coil structure may be the other structural component that contributes to strain hardening in WPI gels, but more research is needed in this area.

With the fracture properties normalized, no relationships were observed with the permeability or the microstructure between treatments. Lower permeability values were found for the treatment values (250 mM NaCl, pH 7 and pH 5.68) near the fine-stranded transition regions ($\sim$ pH 6 and 200 mM NaCl, pH 7). These networks had more of a strand type structure with smaller, less defined aggregates as observed in SEM. This further demonstrates that there is little relationship between fracture rheology and microstructure of particulate gels.

**Electrostatic Effects on the Physical Properties
Of Particulate Whey Protein Isolate Gels**

by

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BIOGRAPHY

Matthew McGuffey was born May 24th, 1975 in East Lansing, MI; to Ken and Pat McGuffey. After living in New Brunswick, NJ and Brookings, SD, at age five his family settled down in New Palestine, IN for the remainder of his childhood. The author has one sibling, a younger sister Lindsey. Matt was very active in sports throughout his early years and in high school played varsity football and basketball. Matt attended Purdue University and was a Food Science major in the School of Agriculture right from the start earning a B.S.-Honors degree in four years. Throughout college, Matt was very active in his Fraternity, Sigma Phi Epsilon, the Food Science Club as well as other organizations. After graduating from Purdue, Matt took one year off from school to work as an intern in the Research and Development Department at Kellogg Co. in Battle Creek, MI. He started graduate studies at NCSU in June of '98 and hopes to continue on for his Ph.D. after the successful completion of his M.S. Matt, his wife Jamie and their daughter, Madison, who was four as of June 23rd, 2000, currently reside in Cary, NC.

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LITERATURE REVIEW

Whey and Whey Proteins

Whey is the fluid release from cheese curd during cheesemaking. The solids in whey can be recovered through many processes, the most prevalent being ultrafiltration and diafiltration (Wong et al., 1996). This can be spray dried to produce whey protein concentrates (WPC) or further processed through ion-exchange and other technologies to produce whey protein isolates (WPI). Protein content of WPC's can range from 35-80%. Whey protein isolates contain >90% protein with the lipids and lactose being largely removed in the process. The varying composition of whey protein products depends on different sources and methods for processing (Morr and Foegeding, 1990).

Since protein comprises such a large fraction of WPI, it is important to understand the molecular structure of the constituent proteins to understand their contribution to functional properties. The four major proteins in whey are β -lactoglobulin, α -lactalbumin, bovine serum albumin, and immunoglobulin, and a typical WPI protein composition is 68%, 17%, 7.5%, and 7.5%, respectively (Morr and Foegeding, 1990). The following few paragraphs provide a general overview of the biochemical characteristics and relevant physical properties of these proteins. Their direct contribution to gel structure and functionality will be discussed in a later section.

Bovine β -lactoglobulin (β -LG) is a globular protein comprised of 162 amino acids, with an isoelectric point of 5.2, that has a molecular weight (MW) of 18,362 for genetic variant A and 18,276 for variant B (Wong et al., 1996). The primary structure of β -LG A and B has been sequenced (Braunitzer et al., 1973). The difference between variants A and B is the substitution of Gly and Ala in β -LG B at the Asp 64 and Val 118 β -LG A positions,

respectively (Wong et al., 1996). The secondary structure of β -LG B has been elucidated as 43% β -sheet, 10% α -helix, and 47% aperiodic (random coil) (Townend et al., 1967; Creamer et al., 1983). Papiz et al. (1986) elucidated the three-dimensional, tertiary structure of bovine β -LG at 2.8 Å. This structure is highlighted by an eight-stranded, antiparallel β -barrel to form the shape of a flattened cone where the interior is rich in hydrophobic amino acids and the opening is lined with hydrophilic amino acids. Two disulfide bonds are positioned at Cys 106 to 119 and Cys 66 to 160 and there is a free sulfhydryl group positioned at Cys 121 that is buried at the interface of the sheet-helix (Papiz et al., 1986; Wong et al., 1996). Pessen et al. (1985) has studied the conformation of β -LG in solution as a function of pH. Between pH 3.5 and 5.2, β -LG exists predominantly in the octamer species. At pH > pI, β -LG dissociates into dimers and at 3.5 < pH < 6.5 the dimers begin to dissociate into monomers due to electrostatic repulsion. In the dimer conformation, the α -helix moiety from each monomer runs antiparallel to the helix of the other (Creamer et al., 1983). There is a single, reversible transition in conformation at pH 7.5 that involves the release of a buried carboxyl group (Tanford et al., 1959). At very alkaline pH (>9.0), the extreme electrostatic repulsion causes β -LG to undergo irreversible denaturation (Wong et al., 1996).

α -lactalbumin is a globular, calcium metalloprotein with a MW of 14,147 for variant A and 14,175 for B (Brew and Grobler, 1992; Wong et al., 1996). The primary structure is known and there are many similarities between α -LA and lysozyme (Nitta and Sugai, 1989). The protein has an ellipsoid shape that has two distinct lobes divided by a cleft where one lobe is comprised of four helices and the other loop is comprised of two β -strands with a loop-like chain (Acharya et al., 1989; Wong et al., 1996). α -LA has no free thiol groups and four

disulfide bonds, which limit the conformational flexibility in certain solvent conditions (Brew and Grobler, 1992).

Bovine serum albumin (BSA) is a rather large globular protein with a MW of 66,411 (based on composition) and 17 double bonds and one free thiol group (Peters, 1996). X-ray crystallography studies on the structurally similar human serum albumin show that it is heart-shaped molecule, 80 Å on each of three sides and 30 Å thick (He and Carter, 1992), with an α -helix content of 67% in 28 α -helix regions (Carter and Ho, 1994). The remainder of the molecule is 23% aperiodic structure and 10% β -turns. The pI of BSA is 5.1 (Kinsella and Whitehead, 1989) with a charge of -17 at pH 7.4 (Peters, 1996).

Immunoglobulin (IgG) has a molecular weight that varies at ca. 160,000 consisting of two heavy chains (MW of ca. 55,000) and two light chains (MW of ca. 25,000) (Larson, 1992). The shape of IgG is more rod-like, being the main non-globular whey protein. The molecule has a Y-shape (Silverton et al., 1977) with two light chains attached to the heavy chains by a disulfide bond and the two heavy chains are attached through a disulfide bond, which gives the molecule conformational flexibility (Larson, 1992). The pI range of IgG₁ and IgG₂ is 5.5-6.8 and 7.5-8.3, respectively (Butler, 1983).

Heat Denaturation of Whey Proteins

The thermal properties of the constituent whey proteins contribute to the functional properties of heat-set gels. The following is a brief discussion on the denaturation/transition temperatures of individual whey proteins in salt/buffer-free conditions at various pH. Then, the effects of pH and salts on the thermal stability of WPC and WPI will be discussed.

The thermal denaturation properties of β -LG have been extensively studied (de Wit and Klarenbeek, 1984; Bernal and Jelen, 1985; Liu et al., 1994). β -Lactoglobulin is the most

heat stable whey protein in acidic and isoelectric conditions with denaturation temperatures of 82°C and 78°C at pH 3.5 and 5.5, respectively (Bernal and Jelen, 1985). This stability diminishes at neutral pH where it denatures at 73.5° and 64.6°C at pH 6.6 and 8.0, respectively (Liu et al., 1994).

α -LA has the lowest denaturation temperatures of the whey proteins, which varies only slightly when pH is varied. The denaturation temperatures are 59°C, 61°C, and 62°C for pH 3.5, pH 6.5, and pH 7.5, respectively (de Wit and Klarenbeek, 1984; Bernal and Jelen, 1985). However, it is the only whey protein that is thermoreversible (>90%) (i.e. it renatures upon cooling) (de Wit and Klarenbeek, 1984).

The denaturation temperature of BSA ranges from 72-74°C through the pH range of 3.5-6.5, making it the second most heat resistant protein behind BLG (Bernal and Jelen, 1985). At pH values less than 3.0, there is no thermal transition indicating the electrostatic repulsion denatures BSA at room temp. Also, BSA is the only whey protein to exhibit a double peak in the intermediate pH range (de Wit and Klarenbeek, 1984; Yamasaki et al., 1991).

There is some disparity in the literature as to the heat stability of the immunoglobulins. Several papers have indicated that IgG is the most heat labile of the whey proteins (Larson and Roller, 1955; Varunsatian et al., 1983). de Wit and Klarenbeck (1984) measured the transition temperature as 77°C at pH 7.5, making IgG the most stable protein at that pH. Recently, Chen and Chang (1998) corroborated the latter study indicating it takes temperatures of >75°C to denature IgG between pH 4 and 10, yet, outside of this pH range IgG was very susceptible to denaturation. This study indicates 60% of IgG is denatured after heating at 85°C for 150 sec (Chen and Chang, 1998).

As expected, the denaturation profile of whey protein concentrate follows β -LG very closely with denaturation temperatures of 88°C at pH 3.5, 79°C at pH 5.5, and 70°C at pH 7.5 (de Wit and Klarenbeek, 1984; Bernal and Jelen, 1985). Recently, Ju and Kilara (1999) reported the onset and denaturation temperatures using DSC of whey protein isolate (WPI) treated at different pH values and increasing CaCl_2 concentrations. Contrary to WPC, WPI exhibits a double transition attributed to α -La at 67°C and BLG at 76°C at neutral pH and with no added salt. However, when salt is added or the pH is lowered from neutral pH, WPI exhibits a single peak. For CaCl_2 (at pH 7), the denaturation temperature decreases to 74°C in 20 mM CaCl_2 , but increases to 78°C in 100 mM CaCl_2 . In the isoelectric region, the denaturation temperature increases from 79.8°C at pH 5.7 to 81.2°C at pH 5.2 (Ju et al., 1999). Xiong (1992) measured the thermal transitions of WPI in NaCl solutions using a turbidimetric method, which is a measure of the protein-protein interactions and aggregation. Untreated WPI shows a transition at 83°C, and when 20 mM NaCl is added, the transition temperature decreases to 75°C, then at 200 mM NaCl, a double transition is measured at 77°C and 86°C corresponding to α -La and β -LG, respectively. These two peaks increase in temperature as NaCl is increased up to 600 mM NaCl, where the corresponding peak temperatures are 79°C and 89° (Xiong, 1992). This indicates that at higher ionic strength, CaCl_2 and NaCl stabilizes the proteins against thermally-induced unfolding and aggregation.

Rheological and Structural Measurement Techniques

Rheology Theory

Rheology examines the response of materials to applied forces and deformations (Daubert and Foegeding, 1998). Fundamental rheological tests are independent of the

instrument used and based on well-defined sample geometries and physically valid equations (Steffe, 1996; Daubert and Foegeding, 1998). Empirical tests are typically instrument-specific and are common with complex sample geometries or when they are correlated with another property of interest. This thesis uses exclusively fundamental tests and that is what will be discussed here.

Two basic concepts of fundamental rheology are stress and strain, which can be divided into normal and shear where the direction of the force with respect to the sample surface is perpendicular and parallel, respectively (Daubert and Foegeding, 1998). Stress (σ) is a measure of force applied over a given area:

$$\sigma = \frac{F}{A}$$

which is typically measured in N/m^2 or Pa. Strain is a dimensionless quantity that measures the relative deformation of the sample in the direction of the force and is given by:

$$\gamma = \frac{\Delta L}{L}$$

The relationship between stress and strain is often presented as a modulus:

$$G = \frac{\sigma}{\gamma}$$

where G takes the units Pa and is a material-specific parameter.

Small and Large Strain Rheological Testing

For solid materials, either small or large strain rheological measurements typically can be made. Small strain rheology refers to measurements made in the linear viscoelastic region of the material (see Figure 1). These tests are typically non-destructive and measure the response of materials over time (creep), temperature (gel cure experiments) or frequency

range (Clark and Ross-Murphy, 1987). Large strain rheology measures the material properties over the entire stress-strain continuum until material failure often referred to as fracture (see Figure 1.). Fracture tests can be run in tension (pulling the sample apart), compression (applying a force perpendicular to the sample) or torsion (twisting the sample).

For β -lactoglobulin gels, small strain measurements can differentiate between but not within the particulate and fine-stranded gel types (Stading and Hermansson, 1990). However, large strain measurements can differentiate gel structural properties within the two gel types (Stading and Hermansson, 1991). For this reason, large strain measurements will be used exclusively in this work.

Torsion Testing

The large strain method of choice for this work is torsion. Torsion utilizes the capstan geometry, which requires a complex series of equations developed by Diehl et al. (1979) to calculate shear stress and shear strain at fracture. In the torsion test, the true shear strain and true shear stress at fracture are calculated from the angular displacement and torque, respectively (Diehl et al., 1979).

The torsion method holds a number of advantages over compression and tension as summarized by Hamann (1991)

1. The test produces pure shear where the specimen volume does not change so poisson's ratio is not involved in the calculations.
2. Specimen shape is maintained during testing, which minimizes geometric considerations.
3. Due to geometry, true shear stress and strain are valid up to 110° twist angles. Beyond, 110° a correction factor is employed up to 300° with reasonable accuracy.

4. It is possible to observe the mode of failure of a specimen since shear, tensile and compressive forces all have equal magnitude but different directions.

5. Friction between the specimen and the test fixture does not have to be considered.

Its major disadvantage relative to compression is the rigorous sample preparation involved, which could cause specimen damage.

Microstructural Techniques

It is possible to probe the microstructure of gels through invasive and noninvasive techniques. In noninvasive techniques, there are no processing steps or chemicals added to prepare the gel for structural evaluation. Permeability (van Dijk and Walstra, 1986; Verheul and Roefs, 1997), small angle x-ray scattering (Clark and Tuffnell, 1980), small angle neutron scattering (Renard et al., 1996) and Raman spectroscopy (Clark et al., 1981b) are all noninvasive techniques that have been used to understand the structure of globular protein gels. The theoretical considerations of the permeability method will be discussed in the following paragraph. Invasive techniques typically employ a microscope to observe the structure of gels from a few hundred microns down to a few nanometers. This review will compare scanning electron microscopy (SEM), transmission electron microscopy (TEM), confocal scanning laser microscopy (CSLM) and light microscopy (LM).

The measurement of the permeability for solid materials have long been made in the fields of groundwater hydrology and petroleum mining (Scheidegger, 1974). The theory has also been extended to chromatography, membrane filtration and deep-bed filtration (Adler, 1989). Only recently has the concept of permeability been extended to protein gels (van Dijk and Walstra, 1986). Darcy (1856) first developed the basic law governing flow through porous media. This work developed a relationship between the rate of fluid flow through a

vertical bed of solid particles and the pressure drop on each end of the bed. This equation has been refined over the years to describe a plethora of matrix phenomena and is referred to as Darcy's Law:

$$v = \frac{-B}{\eta} \Delta P$$

where v is the liquid flux, B is the permeability coefficient (with dimensions of length squared), η is the dynamic viscosity of the flowing liquid and ΔP is the pressure gradient over the fixed matrix (Adler, 1989). This equation is the basis for the determination of the permeability coefficient, which can be calculated through a more complex series of equations (Verheul and Roefs, 1997).

These model equations were developed and are only valid in laminar flow for a Newtonian fluid for an assumed homogenous matrix. The permeability coefficient depends on the geometry, scale, interconnectedness and spatial distribution of the percolated matrix (Scheidegger, 1974).

Microscopic techniques are much more common, but typically require rigorous specimen preparation in order to observe under the microscope. The traditional choices have been SEM, TEM or LM, but more recently, CSLM has become more widely used for the visualization of structures (Corle and Kino, 1996). First, the theory governing both electron microscopic techniques (SEM and TEM) will be discussed and the techniques will be compared and contrasted. Then, the two light-based techniques (LM and CSLM) will be discussed in a similar manner.

Both TEM and SEM use electron beams emitted from a high voltage tungsten filament that are rastered onto a specimen (Bozzola and Russell, 1999). Although there are a variety of signals with various energies that can be emitted by the excited specimen, the two relevant to

this discussion are secondary and transmitted electrons. For secondary electrons to be emitted from the excited specimen, it must first be sputter coated with a thin layer of an electron-rich metal (typically gold and/or palladium). Then, when the specimen is struck by a high-energy electron beam it will emit a lower energy secondary electron (typically 50 eV) (Bozzola and Russell, 1999). These electrons are then captured and projected onto a screen as an image. The advantages of SEM are the ease of sample preparation relative to TEM and the capability to resolve more detail and contour in structures. The SEM is optimally utilized observing structures from ~30 nm up to ~60 μm (Aguilera and Stanley, 1993). To resolve structures below 30 nm, one must turn to TEM as the microscope of choice (Aguilera and Stanley, 1993). For electrons to transmit through the specimen, it must be sliced very thinly ($<1\mu\text{m}$) (Bozzola and Russell, 1999), which requires a great deal of skill. This specimen preparation is one drawback. Also, the microscope has a small depth of focus leading to "flat" looking micrographs that are binary (black and white), so little detail can be resolved. However, since many globular proteins have sizes on the order of 1-10 nm, TEM must be used if fine-stranded protein structures are to be resolved.

The wavelength of electrons accelerated at 60 kV is 5×10^{-12} m (Bozzola and Russell, 1999), which accounts for the high resolution of these instruments. Visible light has a wavelength range from 400 to 800 nm, which explains the relatively lower resolution attained with LM and CSLM (Bozzola and Russell, 1999). A light microscope is used to resolve structures from 900 nm to ~200 μm (Aguilera and Stanley, 1993), but the small depth of focus limits the contour of structures making them appear flat. This is where CSLM has an advantage over, not only LM, but also the electron microscopes as well. The laser is a highly collimated, intense light source that is well suited for z-sectioning, which is required to

produce a 3-D image (Richardson, 1990). This is done by placing a pinhole in the path of the beam, which can be adjusted to move the focal plane above or below the plane of interest (Richardson, 1990; Corle and Kino, 1996). In biological applications, a fluorescent dye-probe often is used that will associate with a structure of interest (usually lipid or protein) (Corle and Kino, 1996). The structure can be observed with minimal specimen preparation, which is another advantage of CSLM over other microscopic techniques.

As has been mentioned, the sample preparation for SEM is quite rigorous, although every step has measures to prevent artifact formation. The first objective is to fix the sample structure to prevent collapse or distortion during subsequent dehydration. The most common protocol for fixing protein gels is to use glutaraldehyde as a primary fixative (reacts with amino groups) and osmium tetroxide as a post fixative (reacts with double bonds) (Bozzola and Russell, 1999). Then, the water in the sample must be removed so the solvent will be compatible with the critical point drying step. Ethanol is typically used in this capacity and is added in a graded ethanol series to slowly increase the concentration, which prevents a sudden shock to the system that might cause artifacts. When the solvent is 100% ethanol, then the specimens are critical point dried using the transitional fluid high pressure CO₂ (Bozzola and Russell, 1999). After the ethanol is bumped out with liquid CO₂, the temperature in the chamber is raised above the critical point of CO₂ and it evaporates leaving a "bone dry" sample (Bozzola and Russell, 1999). Hermansson and Buchheim (1981) determined that critical point drying is the most sensitive step in the entire preparation protocol. Also, it was determined that particulate type gels are less sensitive to the rigors of SEM preparation than fine-stranded gels (Hermansson and Buchheim, 1981). Finally, the samples are sputter coated as described above.

Protein Gel Formation

Almdal et al (1993) reviewed all of the uses of the term gel, correct and otherwise, and summarized three properties a material must possess to be called a gel.

1. At least two components, one of which is a liquid, present in considerable quantity.
2. “A gel is a soft, solid or solid-like material.”
3. A storage modulus $G'(\omega)$ response that exhibits a pronounced plateau at least on the order of seconds, and a loss modulus $G''(\omega)$ that is significantly smaller than the storage modulus in the plateau region.

Flory (1974) classified gels into four categories: ordered lamellar phases (e.g. soap), covalent polymer networks, physically aggregated polymer networks, and particulate disordered networks. Protein gels can take the form of the latter two categories and this thesis deals only with particulate disordered networks.

Ferry (1948b) proposed a general two-step mechanism for heat-induced protein gel formation involving: 1) initiation through unfolding of the protein and, subsequently, 2) an aggregation process between initiated protein molecules (Ferry, 1948b). This process repeats itself until a three dimensional, space-filling network forms. Schmidt (1981) modified this to produce the following set of reactions:

Native Protein $\rightarrow$ Denatured Protein

Denatured Protein $\rightarrow$ Soluble Aggregate

Soluble Aggregate $\rightarrow$ Insoluble Aggregate

Soluble Aggregate $\rightarrow$ Gel

Ziegler and Foegeding (1990) proposed a more complex mechanism for heat-induced gelation specific to whey proteins (Figure 2; diagram taken from (Foegeding et al., 1998)). In

this mechanism, the native proteins aggregate or form a molten globule prior to denaturation. Hirose (1993) characterized the molten globule state as a native-like protein backbone with respect to secondary structure yet denaturation-like alteration of side chains, a slightly expanded hydrodynamic radius, and exposure of hydrophobic clusters in the protein. The denatured aggregates further aggregate through the gel point (as identified rheologically) to an incipient, then cured gel (Ziegler and Foegeding, 1990).

In order to understand the relationship between matrix formation and rheological development, Verheul and Roefs (1998b) studied WPI gels by a permeability method, scanning electron microscopy (SEM) and small strain rheology. It is important to note that they used a temperature (68.5° C) and time (24h) to slow down the kinetics of the gelation process so the structural and rheological phenomena could be observed. They determined that gelation is a two-step process. First, at the gel point (i.e. when a 3-D matrix is formed) the primary spatial structure of the gel is established as determined by permeability and changes little with continual heating. Yet, at this point, the gel has minimal elasticity development, although only ~40% of the protein has been denatured/ aggregated. The elasticity is developed as the remaining ~60% of the protein is incorporated into the matrix “decorating” and reinforcing the pores of the matrix without changing the matrix permeability. Essentially, the mechanism for gel rigidity development is not related to the mechanism of spatial structure development (Verheul and Roefs, 1998b; Verheul and Roefs, 1998a; Verheul et al., 1998). Similarly, Bottcher and Foegeding (1994) found that fine-stranded and particulate gels could be formulated to have similar fracture properties, and Vardhanabhuti et al. observed that gels with similar microstructure have different fracture stress and strain depending on the amount of whey protein polymer included in gels. Apparently, the mechanisms regulating

fracture stress and strain are determined by bonding and interaction energies. Spatial structure is determined by environmental factors, which will be discussed in the next section.

However, it has been noted for ovalbumin (Heertje and van Kleef, 1986) and β -lactoglobulin gels (Stading et al., 1993) that as the network becomes more inhomogeneous (i.e. larger pore size), the fracture stress lowers. This obviously contradicts the current understanding of rheological and structural relationships for globular protein gels over a broad range of network conditions as described in the previous paragraph. In the first study, Heertje and van Kleef (1986) arbitrarily chose their pH values of study as pH 5 and 10. Stading et al. (1993) concluded that the larger pores induced at slow heating rates at pH 5.3 was indicative of a phenomena that could be applied to all gels. Also, neither of the studies discussed potential bonding possibilities in their discussions. This should be mentioned as a precaution when interpreting micrographs.

Gel Network Types

Clark et al. (1981a) observed using transmission electron microscopy that by manipulating the pH and ionic strength of globular protein gels that a variety of microstructures can be obtained. At $\text{pH} > \text{pI}$ with no added salt, BSA gels were macroscopically translucent and had a filamentous structure approximately one protein diameter thick (Clark et al., 1981a). X-ray scattering curves have shown that these gels have a homogenous structure (Richardson and Ross-Murphy, 1981). These X-ray and macroscopic observations indicate the whey proteins have unfolded to a larger extent than the particulate gels formed at different condition (to be discussed below). Monahan et al. (1995) related the unfolding whey proteins to the surface hydrophobicity and demonstrated that protein solutions heated at pH 7, 9 and 11 had increasing surface hydrophobicity. This occurs preferentially when the $\text{pH} > \text{pI}$ because

the disulfide bonds that largely stabilize the protein against unfolding are more reactive at high pH (Monahan et al., 1995). Increased hydrophobicity with protein unfolding is consistent with the common structural feature of globular proteins of the nonpolar residues being located in the protein core to avoid contact with water (Dill, 1990). Clark et al. (1981a) also showed that adding 125 mM NaCl at neutral pH induced a macroscopically turbid gel with a more dense network indicating less repulsion between proteins (the specific causes of the attractive/repulsive forces will be discussed later). When the pH was lowered to the pI, an opaque, phase-separated, coagulate gel was formed that released water when deformed and microscopically had much larger spherical, inhomogeneous structures on the order of 1 μm . In the isoelectric gel, protein repulsive forces have been almost completely removed (Clark et al., 1981a), so the protein will be only slightly unfolded. Richardson and Ross-Murphy (1981) noted similar macroscopic observations for particulate gels and verified through X-ray scattering that the structure is heterogeneous. The macroscopic appearance of particulate gels is caused by the size of the protein aggregates as well as refractive index differences within the gel (Foegeding et al., 1998). Repeated aggregation of partially unfolded globular proteins leads to the characteristic spherical appearance (microscopically) of particulate gels. The translucent, unfolded type of gel has been dubbed a “fine-stranded” gel (Hermansson et al., 1986) and the turbid/opaque, water-releasing gel has been dubbed a particulate gel (Flory, 1974).

Gel Rheological and Structural Factors

The factors contributing to protein gel structure and rheology can be evaluated from many perspectives. The first section will relate the intrinsic factors of globular proteins

mentioned previously to gel rheology and structure. Then, the factors that affect the physical properties of fracture stress, fracture strain, nonlinearity of the stress/ strain curve, and microstructure of globular protein gels will be discussed.

Intrinsic Factors

Many of the intrinsic properties of the constituent whey proteins were described in the opening section. These will now be related to specific gelation properties as they affect rheological and structural properties.

Ferry (1948a) described the relationship between the molecular weight (MW) of polymer (gelatin) comprising the gel network and the gel rigidity. A linear relationship was found between the gelatin MW and the square root of the rigidity (Ferry, 1948a). The ability of most globular proteins to form disulfide bonds (contrary to gelatin) makes this relationship more difficult to establish. Wang and Damodaran (1990) used cysteine and NEM to prevent intermolecular disulfide bond formation (using BSA and soy protein isolate) and they also found a linear relationship ($R^2=0.91$) between protein MW (as determined by SDS PAGE) and square root of gel hardness. Also, they determined that the minimum critical weight averaged MW for globular protein gel formation is $\sim 23,000$. This indicates that the MW is as critical in gel formation as any specific bonding potentials (Wang and Damodaran, 1990). Thus, β -LG (MW of 18,400 kDa) theoretically can not form a gel in fully reduced conditions (i.e. can not form disulfide bonds).

Wang and Damodaran (1991) also studied the effect of protein conformation on the strength of globular protein (BSA) gels. Using circular dichroic analysis on fluid expressed from the gels and low protein concentration solutions, they found that α -helix and aperiodic structure decreased forming β -sheet structure when the protein was heated. As the gel

strength decreased through the addition of salts, there was also a decrease in β -sheet, indicating this might be a major conformational factor contributing to gel strength. They theorized that hydrogen bonded β -sheets acted as junction zones within the gel contributing to gel strength (Wang and Damodaran, 1991). This decrease in α -helix and increase in β -sheet upon formation of BSA gels had been observed elsewhere (Clark et al., 1981b; Yasuda et al., 1986). Although the Clark et al. (1981b) study was not related to gel rheology, the same gel types studied in Clark et al. (1981a), as described in the previous section, were studied. They found that there was little difference in secondary structure between the fine-stranded and particulate gel types (Clark et al., 1981b).

Globular proteins require some unfolding/denaturation event to form a gel (Schmidt, 1981) such as heating (Ferry, 1948b) or addition of a chemical denaturant like urea (Higgins et al., 1951; Xiong and Kinsella, 1990) or guanadine hydrochloride (Egelanddal, 1984). Often, the term's denaturation and aggregation are used interchangeably or in combination to reflect the difficulty in differentiating these two phenomena and their close association. Verheul and Roefs (1998a) measured the concentration of aggregated proteins (relative to native) after a mild heat treatment (68.5°C), and at a variety of pH values at 0.5 M NaCl. When the pH is close to the isoelectric point, the rate of protein conversion from native to denatured is much slower than at neutral pH values. At pH 6, after a 25 hour heat treatment only 20% of the proteins had been denatured/aggregated (Verheul and Roefs, 1998a). This indicates that the lack of electrostatic repulsion at this pH prevents protein unfolding/denaturation. In the same study, Verheul and Roefs (1998a) increased heating temperature up to 80° and 85°C and the proteins denatured almost immediately. Since 80°C is the only heat treatment used in this thesis, care must be taken when comparing these results with

phenomena observed at 68.5°C. To reiterate an important relationship detailed earlier, Verheul and Roefs (1998b) demonstrated that the decrease in native protein concentration upon heating resulted in a subsequent increase in G' (after the gel point), with very little change in permeability.

Liu et al (1994) related the denaturation events using DSC to the onset temperature of gelation for β -lactoglobulin gels. They found that at pH values outside of the isoelectric region ($3.5 < \text{pH} < 6.0$), the course of events was onset denaturation temperature, gelation temperature, then denaturation temperature. Within the isoelectric region, onset gelation temperature occurred prior to or simultaneously with minimum gelation temp (Liu et al., 1994).

Fracture Stress and Elasticity

The fracture stress and elasticity of globular protein gels is strongly affected by a few factors that will be discussed here. First, protein concentration will be discussed. The primary bonding/interactions stabilizing protein gels are disulfide bonds, hydrophobic interactions, electrostatic interactions, hydrogen bonds and van der Waals forces (Schmidt, 1981). Of the aforementioned, disulfide bond formation is the only one that is covalent, and being the strongest, it will be the first factor discussed.

Side Note: Aside from the fundamental tests that detect fracture stress, many empirical fracture studies have been performed that use terms like gel strength and breaking strength. To simplify the discussion, the term fracture stress will be generally used to refer to all of these terms. The term gel hardness will be used distinctly, where applicable, since typically the gel is not fractured when this measurement is employed. Also, the general trends demonstrated with an empirical term will be reported, not specific values.

Protein Concentration

A great deal of research has studied the effects of protein concentration on G' and/or fracture stress response of WPI gels (Foegeding, 1992; Fernandes, 1994; Verheul and Roefs, 1998b; Ikeda et al., 1999), BSA gels (Richardson and Ross-Murphy, 1981; Matsudomi et al., 1994; Tobitani and Ross-Murphy, 1997), ovalbumin gels (Van Kleef, 1986; Arntfield et al., 1990a), and β -LG gels (Paulsson et al., 1990; Matsudomi et al., 1991; Matsudomi et al., 1994). As protein concentration is increased, the number of elastically active network chains also increases (McEvoy et al., 1985), which will obviously increase elasticity (G') and fracture stress. Two studies in particular develop a power law scaling relationship between WPI protein concentration and fracture stress (Foegeding, 1992) and G' (Verheul and Roefs, 1998b). A power relationship takes the form:

$$G' \text{ or } \sigma = A(\% \text{ protein})^n$$

where A is a material-specific constant that is determined by the position of the curve and the exponent n is determined by the slope of the line. For fracture stress, Foegeding (1992) determined values of A and n for 0.05 and 2.63, respectively. Verheul and Roefs (1998b) studied WPI gels treated at 0.1, 0.3, 0.5, 1.0 and 3.0 M NaCl. They determined that all NaCl treatments had similar n -values of ~ 4.5 , and were differentiated by their A -values.

Thiol Activity and Disulfide Bond Formation

The sulfhydryl (SH) group and intramolecular disulfide bonds (SS) present in constituent whey proteins allows them to participate in intermolecular SS bond formation through SH oxidation and/or SH/SS bond interchange reactions, respectively. Disulfide bond formation is known to be a major reaction involved in the denaturation, aggregation (Sawyer, 1968) and gelation (Schmidt et al., 1978) of whey protein systems. To underlie the

importance of the free thiol group in bovine β -LG, Gallagher et al. (1996) compared its gelation to porcine β -LG, which is molecularly similar yet contains no free thiol group. Optimal gelation conditions (10% protein, 200 mM NaCl at pH 7) for bovine β -LG did not produce any G' response in porcine gels (Gallagher et al., 1996). Shimada and Cheftel (1989) studied heat-induced WPI gels (9% protein) and determined that SH/S-S interchange reactions are primarily responsible for the gel elasticity at pH 7.5. For entropy elastic gels, it is expected that when covalent crosslinks are formed, the gel elasticity, G', will increase according to the following equation (Mark, 1993):

$$G' = \frac{cRT}{M_c}$$

where c is the concentration of the elastically active polymer, R is the Universal gas constant, T is the temperature ($^{\circ}$ C), and M_c is the MW of strands between crosslinks. As disulfide crosslinks are formed, the denominator becomes smaller and G' increases.

The activity of the free SH group is strongly affected by pH (Liu et al., 1994; Monahan et al., 1995; Hoffmann and van Mil, 1999). The approximate pKa of cysteine is 9.0-9.5 (Creighton, 1993) so at neutral and alkaline pH, it has been shown that the SH residue is two times more reactive than at acid pH for whey proteins (Dunnill and Green, 1966). However, very few papers have studied the contribution of disulfide bonds to the gelation of whey protein gels in particulate gel inducing conditions. Shimada and Cheftel (1988) studied the pH ranges of 6.5-9.5 and 2.5-3.5, and studied the changes when 0.1M NaCl was added. However, this is not enough salt to induce particulate gels (Foegeding et al., 1998). Recently, Otte et al (2000) evaluated the predominant forces in β -LG gels at pH 3, 5 and 7 with emphasis on disulfide bonds. They determined that at pH 5 disulfide bonds primarily

supported the gel structure. At pH 7, initial aggregate formation was catalyzed mainly by disulfide bond reactions, but these aggregates then associated mainly through noncovalent interactions to form the resultant gel (Otte et al., 2000). Hoffmann and van Mil (1999) demonstrated that there is a dropoff in SH/S-S activity at pH 6.0 when compared to the pH 6.4-8.0 range. Shimada and Cheftel (1989) demonstrated that WPI forms highly elastic gels at pH 7.5 and inelastic gels at pH 2.5, and that SH-induced/S-S interchange reactions were responsible for the gel elasticity at pH 7.5, not SH oxidation reactions. There is very little data on the effect of salts on the reactivity of the thiol group. The closest research out there, for comparison purposes, is Shimada and Cheftel (1988), where they evaluated WPI at pH 6.5-9.5 and 0.1M NaCl. They concluded that adding 0.1M NaCl, decreased water-holding capacity, elasticity and protein solubility, while increasing gel firmness at all pH values.

There are many studies that have used thiol reagents to determine the effect of disulfide bonds on structural and functional properties of globular proteins. Numerous studies have shown that adding an excess molar quantity of the thiol reagents dithiothreitol (DTT) (Matsudomi et al., 1991; Errington and Foegeding, 1998), n-ethylmaleimide (NEM) (Shimada and Cheftel, 1988; Doi et al., 1990; Wang and Damodaran, 1990; Matsudomi et al., 1991; Sato et al., 1995), cysteine (Schmidt et al., 1979; Wang and Damodaran, 1990), or β -mercaptoethanol (BME) (Doi et al., 1990; Mulvihill et al., 1991) dramatically reduces fracture stress or gel hardness of globular protein gels. Cysteine and NEM react directly with the free SH group whereas DTT and BME reduce disulfide bonds to SH groups (Creighton, 1993). The thiol blocker NEM is used in this thesis, so the following information will be useful. Recently, Hoffman and van Mil (1997) studied the role of disulfide bonds in β -LG aggregation at pH 7 with no added salt. β -LG solutions were heated 30 hours at 65°C at

molar ratios of 0-6 NEM/BLG (1 BLG = 1 free thiol). From this, they determined that up to a molar ratio of one, NEM reacts preferentially with the free thiol group, not any SH/SS interchange products. This corresponded to a dramatic decrease in peak temperature and enthalpy as determined by DSC. Beyond the molar ratio of one, these curves reached a plateau value (Hoffmann and van Mil, 1997). Xiong et al (1993) surmised that blocking the free thiol group of β -LG (i.e. prevention of intermolecular crosslinking) increased the flexibility of the molecule, which enhances other possible interactions (hydrophobic, hydrogen, etc.).

Hydrophobic Interactions

A typical structural feature of globular proteins is a core rich in hydrophobic amino acids that prevents penetration by water (Dill, 1990). Protein unfolding as a result of SS/SH interchange reactions (usually after heating) can expose these hydrophobic regions from the interior of the molecule enhancing their participation in aggregation and gelation (Shimada and Cheftel, 1988; Li-Chan and Nakai, 1990). Hydrophobic interactions are thermodynamically favored at high temperatures because the ordered structure of water present at room temperature becomes disordered and takes on higher energy states when the system is heated (Dill, 1990). It has been suggested that at pH values of 6.0 and lower that hydrophobic interactions predominate, and contribute a great deal of strength to WPI gels (Harwalkar and Kalab, 1985; Zirbel and Kinsella, 1988; Sato et al., 1995) and other globular protein gels (Hayakawa and Nakai, 1985; Arntfield et al., 1990b; Tobitani and Ross-Murphy, 1997). Beveridge et al (1984) have noted that gels formed primarily through hydrophobic interactions are inelastic whereas gels formed through intermolecular disulfide bond formation are elastic in nature. Relkin (1998) concluded that when β -LG was heated at 80°C

that thiol-disulfide interchange reactions and hydrophobic interactions could be "superimposed" in terms of their effect on the sol-gel transition.

Electrostatic Interactions and Salt Effects

Right after disulfide bonds and hydrophobic interactions, in order of contribution to structure, electrostatic interactions play a major role in the folding of proteins, which effects the elasticity and strength of globular protein gels. The pH dictates the total charge on the protein whereas the ionic strength determines the degree of interaction among the charges because salts interact with charges (Dill, 1990). Since both of these environmental factors affect the degree to which proteins will interact with one another, they will obviously affect gel elasticity and strength.

The small strain rheology of β -LG gels as a function of pH have been well studied (Paulsson et al., 1990; Stading and Hermansson, 1990). Both studies found a G' maximum in the isoelectric region relative to acidic and neutral pH values and Stading and Hermansson (1990) determined that the minimum protein concentration to form a gel at pH 5.5 is 1% . Stading and Hermansson (1991) also evaluated the large strain properties of β -LG gels at varying pH. Two regions could be differentiated with fracture stress within the isoelectric region. Between pH 4.0-5.2, fracture stress values remain relatively constant at ~ 3 kPa, but jump up to ~ 9 kPa at pH 5.5 before declining slightly at pH 5.75 (Stading and Hermansson, 1991). It has been previously observed that pH 5.5 seems to be an optimum for protein-protein interactions (Ziegler and Foegeding, 1990), which may explain the maximum observed by the previous studies.

When added to protein solution, monovalent salts will shield charges on the protein. This reduces the electrostatic repulsion on the protein and will prevent it from fully unfolding,

which at neutral pH will result in particulate gel formation (Foegeding et al., 1998). This charge shielding phenomena is mainly valid at low salt concentrations (Dill, 1990). At higher concentrations, the charges on the protein are saturated and the salt effects are on the solvent properties (either dielectric constant (Dill, 1990) or solvent quality). Two studies have demonstrated similar trends in fracture stress of β -LG gels (Mulvihill and Kinsella, 1988) and WPI gels (Kuhn and Foegeding, 1991) with 0-1M added NaCl and 0-200 mM CaCl_2 . For NaCl, the fracture stress increases dramatically up to ~100 mM, then plateaus to 200 mM, then decreases as more NaCl concentration is increased (Mulvihill and Kinsella, 1988; Kuhn and Foegeding, 1991). A G' maximum has been observed for WPI gels around 0.1-0.2 M NaCl with a one order of magnitude decrease when NaCl concentration was increased to 1.0 M (Verheul and Roefs, 1998b; Ikeda et al., 1999). These phenomena match up nicely with the molecular occurrences previously noted by Dill (1990). As NaCl conc. is increased up to 0.1-0.2 M, the ions primarily interact with the charges on the protein, which enhances the interactions between proteins. Beyond this concentration, the steady decrease in fracture stress/elasticity is probably caused by changing the solvent properties. This latter observation has also been made by Verheul et al (1998b).

Divalent salts have the charge shielding properties of monovalent salts and can also participate in bridging between adjacent charges on the protein (Schmidt et al., 1979). For CaCl_2 , there is a slight discrepancy in results. Kuhn and Foegeding (1991) show a fracture stress maximum at ~25 mM whereas Mulvihill and Kinsella (1988) show a maximum at 15 mM (measuring stress at 70% compression). Zirbel and Kinsella (1988) showed a gel hardness maximum at 20 mM CaCl_2 for WPI. The β -LG preparation used by Mulvihill and Kinsella had 2% NaCl, which might have accounted for the difference in results or else the

obvious difference in proteins present. Beyond each studies maximum, the stress decreases only slightly to 0.1 M. When compared on an equal ionic strength basis, NaCl and CaCl₂ have very similar trends for stress although NaCl declines from its maximum to lower fracture stress values than CaCl₂ (Kuhn and Foegeding, 1991). Since ionic strength is a measure of the charge concentration, the similar behavior of fracture stress at low ionic strength is probably due to charge shielding. The higher stress at high CaCl₂ conc. (relative to NaCl) is probably due to calcium bridging. The big difference between the two salt types in their effect on WPI gels lies in their strain, which will be discussed later.

Hydrogen Bonding

The last interaction to be discussed is hydrogen bonding. Hydrogen bonds are weak relative to other interactions. Although native structure contains a great deal of hydrogen bonding, this is generally disrupted as the heat-induced gels are formed since hydrogen bonds are thermodynamically disfavored at high temperatures (Dill, 1990). Beveridge et al (1984) related the large increase in G' upon cooling of WPC gels to an increase in hydrogen bonding. Certainly, hydrogen bonding plays some role in the development of structure in heat-induced globular protein gels, however, the difficulty in measuring hydrogen bonding, has hindered the understanding of its contribution to structure.

Fracture Strain (Deformation)

The mechanisms that regulate strain are not as well understood as those previously discussed with regards to stress. It has been noted that as deformation becomes large for elastomers, the structure of the polymer becomes increasingly non-Gaussian (McEvoy et al., 1985). For particulate gels, as deformation occurs the particles themselves as well as the bonds will bend (Bremer, 1992), until a critical strain level is reached, where the material will

fracture. Also, it has been suggested that smaller flocs or aggregates of protein are more deformable than large aggregates (Ikeda et al., 1999).

Stading and Hermansson (1991) reported that fracture strain remains constant throughout the particulate pH region of 4-6. The strain is also relatively small compared to the fine-stranded region at pH 7 (Stading and Hermansson, 1991). This indicates that as the protein unfolds in fine-stranded gels, the gel network strands can slide past one another more readily than when the gel is formed by spherical particles (i.e. particulate gels).

As noted before, the fracture strain is very different between mono- and divalent salts (Kuhn and Foegeding, 1991). For NaCl, the shear strain starts high at ~ 2.6 then quickly decreases to ~ 1.4 for 150 mM NaCl. CaCl_2 shows the opposite trend, starting low at ~ 1.0 with 7.5 mM CaCl_2 , but quickly increases to 2.5 with 30 mM CaCl_2 (Kuhn and Foegeding, 1991). There has not been a reasonable explanation given for this phenomenon in the literature and one will not be proposed here.

Non-Linearity of the Stress/Strain Curve

Often, the strain and stress do not increase in a linear relationship; this non-linear relationship and its mechanism will be discussed in this section. The stress/strain curve can deviate from linearity either with an r-shaped curve (strain softening) or a J-shaped curve (strain hardening as in Figure 1).

Stading and Hermansson (1991) have been the only group to study the non-linear behavior of the fracture curve for particulate whey protein (β -LG) gels. This study fit a power law equation to the stress/strain curve and interpreted the exponent as the degree of linearity. An r- and a J-shaped curve would give a value <1 and >1 , respectively. The calculated 'p-value' (exponent) was ~ 0.8 with large standard deviations from pH 4-5. Starting at pH 5.2,

the p-value was ~ 0.77 , but had a very small std. dev. The p-value steadily increased to ~ 0.95 as pH was increased up to six with a continuing small std. dev (Stading and Hermansson, 1991). Li et al. (1999) studied WPI and egg white gels in fine-stranded conditions. The non-linearity of the stress/strain curve was calculated as a rigidity ratio, where instead of modeling the entire stress/strain fracture curve, the fracture modulus, G_f , is divided by the modulus at 30% strain, $G_{0.3}$, to get the rigidity ratio or slope ratio. There are two items of interest in this study. First, although conditions favored fine-stranded gel formation, it should be noted that the fracture modulus and the rigidity ratio exhibited a power law scaling for both protein types (Li et al., 1999). It is not known if this relationship is valid for particulate gels. Also, this method for determining the non-linearity of the stress/strain curve will be used in this thesis so it is of note.

Recently, a phenomenological evaluation of this non-linear behavior in gelatin gels has been carried out (Bot et al., 1996; Groot et al., 1996). Gelatin may be a good model for comparison to globular protein gels because both gel types have similarly shaped strain hardening curves. Groot et al. (1996) experimentally verified that strain hardening in gelatin gels was best explained by two models: 1) the fractal dimension of the polymer strands; 2) the presence of both stiff rods and flexible coils (Groot et al., 1996). At a molecular level, Bot et al. (1996) interpreted these results in terms of the structure of gelatin. Gelatin gels are comprised of both helical cross-links and random coil structure. As the cross-links grow in number, the strain hardening of the stress/strain curve is amplified (Bot et al., 1996). To extrapolate these results to globular protein gels, this phenomenon can be generalized as follows. Most gels typically have more than one structural entity. As the non-linearity of the stress/strain curve increases, the presence of another gel structural element is increasing.

Especially since the mechanisms of strain are not well understood, the phenomena of non-linear stress/strain behavior is very complex and must be approached cautiously.

Microstructure

The two primary methods that have been used to evaluate the microstructure of globular protein gels are permeability and microscopy with the latter receiving the most attention. The following paragraphs will highlight the studies that have evaluated the microstructure of particulate WPI and β -LG gels.

The most comprehensive and relevant study of the microstructure of particulate β -LG gels as a function of pH was executed by Langton and Hermansson (1992). They evaluated gels treated at pH 4.0, 4.5, 5.5, and 6.0 with SEM and TEM. In the pH region around 4.5, the network is irregular with large pores, whereas in the region from pH 5.0-5.5 the network was more regular (Langton and Hermansson, 1992). This thesis deals exclusively with the pH range from pH 5-6, so this is what will be detailed here. Within the pH 5-6 range, Langton and Hermansson (1992) found the network from pH 5.0-5.5 to be made up of larger particles on the order of 1 μ m. When the pH is increased beyond 5.5, the particles become smaller, typically <0.5 μ m. Whey protein concentrate gels were compared to β -LG gels and the microstructures were quite similar in appearance. It was concluded that β -LG dictates the gelation properties of WPC (Langton and Hermansson, 1992). Briefly, Stading et al (1993) demonstrated that when pH 5.3 β -LG gels were heated at slow and fast heating rates that they exhibited heterogeneous structure with large pores and a homogenous structure with small pores, respectively (Stading et al., 1993).

Clark et al (1981a) first demonstrated that adding NaCl at neutral pH elicits a particulate structure with large aggregates relative to a neutral pH gel without salt. Mulvihill

and Kinsella (1988) demonstrated with TEM that as salt (CaCl_2) concentration is increased, the microstructure becomes more heterogenous and the pore sizes become larger. Similarly, Verheul and Roefs (1998b; 1998a) demonstrated using SEM, CSLM and permeability that as NaCl concentration is increased, the structure becomes more coarse with increasingly large pores. In their subsequent work, Verheul et al (1998) proposed that initially a fractal network comprised of 'building particles' is formed that, upon continued heating, has its pores 'decorated' by the remaining protein, which reinforces the gel structure.

FIGURE 1– SCHEMATIC REPRESENTATION OF SMALL STRAIN BEHAVIOR IN THE LINEAR REGION AND LARGE STRAIN BEHAVIOR OVER THE ENTIRE FRACTURE PROFILE

Chemical Mechanism: β -Lactoglobulin

FIGURE 2. POSSIBLE CHEMICAL MECHANISMS FOR β -LACTOGLOBULIN GELATION INCLUDING RHEOLOGICAL DEVELOPMENT.

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INTRODUCTION

Whey protein isolates (WPI) are utilized in meat, dairy and bakery products for their gelation, emulsification and foaming properties. Whey protein isolate is comprised of β -lactoglobulin (β -LG), α -lactalbumin, bovine serum albumin (BSA), and immunoglobulins (Morr and Foegeding, 1990). The rheology (Hines and Foegeding, 1993) and microstructure (Langton and Hermansson, 1992; Verheul and Roefs, 1998b) of WPI gels is strongly related to functional characteristics of β -LG. The functional properties of heat-induced WPI or β -LG gels are strongly related to protein concentration (Foegeding, 1992; Fernandes, 1994; Verheul and Roefs, 1998b; Ikeda et al., 1999), pH (Stading and Hermansson, 1990, 1991; Langton and Hermansson, 1992), ionic strength/ salt type (Mulvihill and Kinsella, 1988; Kuhn and Foegeding, 1991; Bowland and Foegeding, 1995; Kinekawa et al., 1998), and heating temperature/rate (Stading et al., 1993; Liu et al., 1994; Verheul and Roefs, 1998a). In sufficient concentration, globular proteins can form fine-stranded, mixed or particulate gels depending on pH and ionic strength (Clark et al., 1981; Foegeding et al., 1998). Clark et al. (1981) demonstrated that when BSA gels were heated at their isoelectric point (pI) with no salt added or at pH 6.5 with 0.125 M NaCl, that gels with larger aggregate structures were formed. Particulate gels are particularly relevant to study because they are formed under pH and/or salt levels typical of many food products.

Verheul and Roefs (1998a) slowed the kinetics of gelation by heating WPI solutions at 68.5°C for 20 hours and were able to separate spatial structure and gel elasticity development. Gel spatial structure is determined by the concentration of aggregated protein at the gel point and gel elasticity is related to the amount of protein incorporated after the gel point (Verheul and Roefs, 1998a). This reinforces recent observations that rheological properties are largely

unrelated to the microstructure of WPI gels (Vardhanabhuti et al., ; Bottcher and Foegeding, 1994). The rheological properties of gels seem to be related to the bonding/ interactions between the proteins, which cannot be evaluated through microstructural techniques.

The whey proteins β -LG and BSA both have one free thiol group and 2 and 17 disulfide bonds, respectively. Disulfide bond formation is known to be a strong contributor to gel texture in WPI gels (Shimada and Cheftel, 1988; Monahan et al., 1995), and the thiol activity is strongly pH-dependent (Liu et al., 1994; Hoffmann and van Mil, 1999) due to the pKa of cysteine, which is ~ 9.0 - 9.5 in proteins (Creighton, 1993). Recently, Otte et al. (2000) demonstrated that disulfide bonds mainly supported the gel structure of β -LG gels at pH 5, whereas, at pH 7, disulfide bonds catalyzed initial aggregate formation, which facilitated noncovalent interactions to form the resultant gel.

Large strain rheological techniques have been shown to be more sensitive to structural differences in particulate β -LG gels (Stading and Hermansson, 1991) than small strain measurements, and will be used exclusively in this study. Often, the stress and strain do not increase in a linear manner, either hardening or softening with increasing strain. Recently, a phenomenological evaluation of strain hardening in gelatin gels has provided a possible structural interpretation of this behavior (Bot et al., 1996; Groot et al., 1996). The strain hardening was best explained experimentally by two models: 1) the fractal dimension of the polymer strands; 2) the presence of both stiff rods and flexible coils (Groot et al., 1996).

The objective of this research was to compare the functionality of particulate WPI gels formed by adding NaCl at neutral pH and lowering the pH to near the isoelectric point. The approach was to determine if gels with similar fracture properties could be produced by the two different treatments and to evaluate the microstructure through permeability, water-

holding capacity and scanning electron microscopy (SEM). Although, Bottcher and Foegeding (1994) could not correlate any fracture properties to microstructural factors for WPI gels, they evaluated multiple salts, varying protein concentrations and both particulate and fine-stranded gels. It has been noted that gels formed by lowering the pH to near the pI have a similar microstructure to gels formed by adding NaCl at neutral pH (Langton and Hermansson, 1992) and these two treatments are "exchangeable physical parameters" with regards to spatial structure (Verheul and Roefs, 1998a). This work will clarify the similarities between both fracture properties and microstructure and determine if these gels are formed by similar mechanisms.

MATERIALS AND METHODS

Materials

Whey protein isolate (WPI)(tradename BiPro[®], Lot # LE 033-8-420) was obtained from Davisco Foods International (Le Sueur, MN). The powder contained 0.13% potassium, 0.11% calcium, 1.25% sulfur, 0.53% sodium, 345 ppm phosphorous, and 130 ppm magnesium, as determined by inductively-coupled plasma atomic emission spectroscopy. The powder contained 14.36% nitrogen as determined by the Dumas combustion method (AOAC method # 968.06) (AOAC, 1995) and protein content was calculated to be 91.6% (N x 6.38). All chemicals used were analytical or SEM grade.

Gel Formation

All gels were formulated to be 10% protein (w/v). There were two general types of treatments: 1) lowering the pH to near the isoelectric point of WPI ("pH treatment"); and 2) adding NaCl at pH 7.0 ("NaCl treatment"). Dispersions of WPI were prepared by hydrating

the powder with ~90% of the total volume with deionized (DI) water ($>17\text{ M}\Omega$) and/or the appropriate amount of NaCl solution. The dispersions were stirred for one hour, then 1.0 N HCl or NaOH was added dropwise to adjust the pH to 7.00 ± 0.01 for NaCl treatments and to the exact pH for pH treatments. Dispersions were diluted to volume then degassed for one hour in a vacuum dessicator at -86 kPa . The dispersions were added to stoppered glass tubes (19 mm in internal diameter), which were precoated with Sigmacoat (Sigma, St. Louis, MO) and covered with aluminum foil to minimize evaporation. Gels were formed by heating in an 80°C water bath for 30 minutes. After heating, gels were cooled to $22 \pm 1^\circ\text{C}$ for one hr prior to overnight storage at 4°C . The next day gels were equilibrated to $22 \pm 1^\circ\text{C}$ for one hr prior to analysis.

Fracture testing

The torsional fracture method of Kim et al. (1986) was used to measure true shear stress at fracture (fracture stress) and true shear strain at fracture (fracture strain). Cylindrical gels samples were cut to 28.7 mm in length, and each end was mounted onto plastic discs with cyanoacrylate glue (Super Glue, Loctite Corp., Cleveland, OH). The cylinders were ground to a capstan shape with a center diameter of 10 mm (Montejano et al., 1983) using a precision milling machine (model GC-TG92 US, Gel Consultants, Raleigh, NC). Samples were twisted at 2.5 rpm to failure on a Torsion Gelometer (Gel Consultants, Raleigh, NC) and fracture stress and fracture strain were calculated from torque and angular displacement, respectively, as described by Diehl et al. (1979). The fracture rigidity (G_f) was calculated as fracture stress/fracture strain. The rigidity ratio ($R_{0.3}$) was calculated as the ratio of G_f to the rigidity at 30% strain as shown in figure 1.

Water holding capacity

The water holding capacity (WHC) of the gels was determined by the microcentrifuge-based method of Kocher and Foegeding (1993). A sharpened metal tube was used to bore out cylindrical gel samples 10 mm in length and 4.8 mm in diameter. The gels were inserted into microcentrifuge filtration units (Lida Corporation, Kenosha, WI, USA) and spun at 153 X g for 10 minutes in a Beckman Microfuge® 11 horizontal rotor microcentrifuge (Beckman Instruments, Inc., Palo Alto, CA, USA). The moisture content remaining in the gels was determined using a modified AOAC method (AOAC, 1995). Samples were weighed into aluminum pans and dried at 70°C, -92 kPa for 4 hours, then cooled for >12 hours in a dessicator prior to final weighing and moisture determination. WHC values were calculated as a ratio of weight of water held in gels after spinning to weight of protein in the gels.

Permeability

A modified procedure of Verheul and Roefs (1998b) originally by van Dijk and Walstra (1986) was used to determine the permeability of the gels. This method uses a differential solvent level to apply a pressure gradient across a fixed matrix and measures the rate of unidirectional solvent flow (see Figure 2). The liquid flux, v , (i.e. solvent flow rate) obeys Darcy's law in laminar flow conditions for a homogenous matrix. Darcy's law takes the form:

$$v = \frac{-B}{\eta} \Delta P \quad (1)$$

where v is the liquid flux (volume flow rate/cross-sectional area)(m/s), B is the permeability coefficient (m²), η is the dynamic viscosity of the flowing liquid (Pa· s) and ΔP is the pressure

gradient over the fixed matrix (Pa/m) (Verheul and Roefs, 1998b). The liquid flux can be represented as:

$$v(t) = \frac{dh(t)}{dt} \quad (2)$$

and the initial pressure gradient over the gel is given by:

$$\frac{dP(t)}{dx} = -\rho g \frac{(h(\infty) - h(t))}{H} \quad (3)$$

where ρ is the density of the solvent (kg/m^3), g is the gravitational acceleration (m/s^2), $h(\infty)$ and $h(t)$ the height of solvent in the reference tube and the gel tube, respectively (see Fig.2).

Substituting Equations (2) and (3) in Eq. (1) and integrating from $t = 0$ to $t = t$ gives:

$$B_{gel}t = -\ln \left[\frac{h(\infty) - h(t)}{h(\infty) - h(0)} \right] \frac{v(H)}{g} = \Gamma(t) \quad (4)$$

When the right hand side of the equation is plotted vs. time, the slope of the best-fit line is calculated to be B_{gel} .

The procedure for gel formation was followed identically except precision glass tubing (i.d. = 3.89 mm, L = 21 cm)(Ace Glass, Vineland, NJ) was inserted into larger stoppered glass tubes holding the WPI dispersion. The tubes were filled to ~8 cm in height prior to heating at 80°C for 30 minutes. Prior to permeability measurements, the gelled tubes were removed from the surrounding gel and the exterior of the tubes was cleaned. For each treatment replicate, three gelled tubes along with one reference were inserted into a large glass cylinder. The glass cylinder was filled with the solvent used to hydrate the WPI. The medium solvent was used to wet the surface of each gel tube. The height of the solvent as it traveled up through the gel tube, $h(t)$, was measured with a cathetometer (Eberbach Inc., Ann Arbor, MI). The time interval between measurements depended on the permeability of the gel matrix.

Microscopy

Sample Fixation and Dehydration

A modified procedure of Hermansson et al. (1986) was used for fixing and dehydration of the gel samples. Approximately 2 X 3 X 3 mm specimens were cut from the interior of the gel cylinder. To prevent osmotic effects, all fixation and rinse steps were buffered in 0.1M Na₂PO₄ (pH 7.0) for NaCl treatments and 0.025M Na₂PO₄ (pH 5.8) for pH treatments. After both fixation steps, samples were rinsed with the appropriate buffer three 20 min exchanges at 4°C. Treatments were fixed in a 3% glutaraldehyde (EMS, Fort Washington, PA) solution for 2 hr in a 4°C icebath. The gel specimens then were post-fixed in an appropriately buffered 1% osmium tetroxide solution overnight in a 4°C ice bath. Specimens then were dehydrated in a graded ethanol series (30-50-70-95-100-100) at 4°C for 20 min. each. The second 100% dehydration was allowed to sit at room temperature for 20 min. and, finally, room temperature 100% ethanol was added and allowed to sit for 20 min. The samples then were immediately critical point dried.

Critical Point Drying and Sputter Coating

Samples were transferred to a compartmentalized wire mesh basket with care being taken to maintain sample contact with 100% ethanol to prevent air-drying. The basket was placed in the critical point dryer (Tousimis, Rockville, MD) reservoir and the chamber was sealed. The chamber temperature was lowered to ca. 0°C and then filled with liquid CO₂ to purge the chamber of ethanol. The temperature was raised to >40°C and subsequently the pressure raised to 12-1400 psi to convert the liquid CO₂ to vapor, which is then bled off, resulting in a bone dry sample. The dried samples then were carefully fractured using a razor blade and

tweezers, and mounted onto stubs with colloidal silver paint (EMS, Fort Washington, PA). The silver paint was allowed to dry for 24 hours in a vacuum dessicator.

Next, the specimens were coated with $\sim 25\text{\AA}$ of Au/Pd ($10\text{\AA}$ with samples at 45° angle and $15\text{\AA}$ with samples at 90°) in a sputter coater (Hummer V, Technics EMS, Inc., Springfield, VA). Specimens were held in a vacuum-dessicator until viewing at 10-15 kV.

Statistical Analysis

Significant differences were determined using the Data Analysis package for Excel (Microsoft Co., Redmond, WA). A single factor ANOVA was performed ($\alpha=0.05$) in comparing treatments and values were reported as not significantly different at $p<0.05$ or $p<0.001$.

RESULTS

In order to find isostress and isostrain pairs between treatments, the fracture stress and fracture strain were plotted throughout the relevant treatment range. The fracture stress and fracture strain profiles for particulate gels, throughout the relevant treatment range, previously have been reported for both pH (Stading and Hermansson, 1991) and NaCl treatments (Mulvihill and Kinsella, 1988; Kuhn and Foegeding, 1991). For fracture stress, Figure 3 demonstrates a parabolic shaped curve from pH 5.2-5.8 and a maximum at pH 5.5. Fracture strain increases from pH 5.2 to 5.5, then remains constant from pH 5.5 to 5.8 (Figure 4). Although the numbers are different, probably due to the difference in methods used, the trends are largely the same as found by Stading and Hermansson (1991). For NaCl treatments, fracture stress has a maximum at 0.2 M NaCl (pH 7) and gradually decreases through 0.6 M. Fracture strain increases from 0.2 to 0.3 M, then decreases through 0.6 M.

These results generally follow those from Kuhn and Foegeding (1991), but are difficult to verify since that work did not test points between 0.2 and 0.6 M NaCl. When fracture stress (Figure 3) and fracture strain (Figure 4) curves for both treatments are overlaid, similar trends can be seen, which has not been previously reported. The fracture properties of gels treated with CaCl_2 were also overlaid (data not shown), but the much higher strain in these gels as reported by Kuhn and Foegeding (1991) prevents this data from overlaying with pH and NaCl data. From this data, two treatment pairs were formulated at high stress (pH 5.47 and 0.25 M NaCl, pH 7.0) and low stress (pH 5.68 and 0.6 M NaCl, pH 7.0) (Table 1). Each pair is not significantly different (NSD) ($p>0.05$) for fracture stress and there is no significant difference between any of the treatments for fracture strain. There is ~10 kPa difference between the high stress and low stress pairs. The only significant difference is the fracture stress between pairs ($p<0.001$).

All gels tested for both treatments demonstrate a strain hardening relationship ($R_{0.3}>1$)(Figure 5). Interestingly, the rigidity ratio ($R_{0.3}$) is treatment specific, not pair specific (see Table 1). Indeed, Stading and Hermansson (1991) conclude that non-linearity of the stress/strain curve does not influence the fracture stress for β -LG gels. The gels from 0.25 and 0.6 M NaCl (pH 7) treatments are NSD ($p>0.05$) for $R_{0.3}$. The gels treated at pH 5.5 and 5.68 are significantly different ($p<0.05$). Strain hardening increased as the pH is increased above 5.5 up to pH 5.8, where $R_{0.3}$ is similar to the values obtained for the gels from the NaCl treatment (Figure 5). Although Stading and Hermansson (1991) demonstrated a strain weakening relationship ($R_{0.3}<1$) for particulate β -LG gels in the isoelectric region, their general trend demonstrated an increasing value as the pH increased from 5.2 to 6.0. Also, their values were only slightly less than one indicating that their values were close to the

values obtained in this study. The difference in values can be reconciled against this study either through their 12% protein concentration (compared to 10% used here), their use of a pure protein, and/or their measurement of fracture properties in tension.

Since the fracture properties have been normalized for both treatments, the isopairs can be evaluated to determine if there is a common relationship with the microstructure.

Permeability, water-holding capacity and SEM methods were used to evaluate the microstructure of the gels.

The permeability coefficient depends on the geometry, scale, interconnectedness and spatial distribution of the percolated matrix (Scheidegger, 1974). For all treatments, when $\Gamma(t)$ vs. 't' was plotted to obtain a value for B_{gel} , a straight line ($r^2 > 0.99$) was obtained. Verheul and Roefs (1997) demonstrated that B_{gel} does not change as the initial pressure gradient (eq. 3) is varied from 3 to 22 kPa/m, which validates Darcy's law. In our work, the initial pressure gradient ranged from 3.0 to 8.7 kPa/m, which falls well within the valid working range established by Verheul and Roefs. Furthermore, the liquid flux ranged from 10^{-5} to 10^{-9} m/s, which is sufficiently low to maintain laminar flow.

Verheul and Roefs (1998b) determined that the permeability (B_{gel}) of particulate WPI gels increases as NaCl concentration is increased. Table 1 demonstrates that as NaCl is increased from 0.25 M to 0.6 M, B_{gel} increases $\sim 0.9 \times 10^{-13} \text{ m}^2$. These numbers generally agree with those generated by Verheul and Roefs (1998b). They calculated a B_{gel} of $\sim 6 \times 10^{-14} \text{ m}^2$ at 0.25 M NaCl (pH 6.8) when heated at 68.5°C and in a subsequent study (Verheul and Roefs, 1998a) found that increasing the heating temperature to 80°C increases the B_{gel} one order of magnitude. This inferred value, $\sim 6 \times 10^{-13}$, compares favorably with the $\sim 2 \times 10^{-14}$ value given in Table 1. Any discrepancy between values can be explained by extrapolation

of their values to higher protein concentration and interpolation between 0.2 and 0.3 M NaCl as well as experimental differences. For the pH treatments, for which permeability results have not been previously reported, B_{gel} decreases by one order of magnitude as the pH is increased from 5.47 to 5.68.

Bowland and Foegeding (1995) have shown that increasing levels of salt cause decreases in water-holding capacity (WHC). Table 2 results verify this observation demonstrating that when NaCl is increased from 0.25 to 0.6 M (pH 7), the WHC is significantly different ($p < 0.05$). The results from the pH treatments also demonstrate that the treatments are significantly different ($p < 0.05$). The trend however is opposite of what would be expected based on permeability results. The WHC method is an indicator of the pore size of the gels, which allows them to release fluid when deformed. The larger pores, as indicated by permeability and SEM, seen at the pH 5.47 treatment should yield a lower WHC than at pH 5.68 where the pores are much smaller. This indicates that the WHC probably is influenced by gel strength (as indicated by fracture stress). Gel cylinders used for the method with lower stress do not have as much strength for support so they express more water than gels with higher stress. The permeability method seems to be more sensitive to gel microstructure than the WHC method.

The micrographs corroborate both the permeability results and previous research. For NaCl treatments (Figure 6), as NaCl concentration was increased from 0.25 M to 0.6 M, pore size increased as noted by Verheul and Roefs (1998b). This corresponded to a large increase in B_{gel} (Table 2). Protein aggregates appeared to be ~ 0.1 - $0.4 \mu\text{m}$ in diameter and the surface of the aggregates were not well defined or smooth. This is consistent with observations made by Langton and Hermansson (1996) who executed a stereological study and determined that

WPI gels treated at pH 5.4 (no salt) were observed to be smooth and the same gel with 0.1 M NaCl had a "hairy" appearance. For pH treatments (Figure 7), at pH 5.47 the aggregates were $\sim 0.9\ \mu\text{m}$ in diameter with quite large pores, but when the pH was increased to 5.68 the particles became much smaller ($\sim 0.2\ \mu\text{m}$) and the network became less porous. Langton and Hermansson (1992) observed a similar transition in microstructure for β -LG gels formed at pH 5.5 and pH 6.0. This transition corresponded to a large decrease in B_{gel} (Table 2).

DISCUSSION

Particulate WPI gels from pH and salt treatments demonstrate very similar fracture stress and fracture strain profiles over their treatment ranges. This indicates that there may be a common mechanism for formation of particulate gels, regardless of treatment. This has not been previously reported as Verheul and Roefs (1998a) studied the effects of adjusting pH from 7.5 down to 6 in 0.5 M NaCl, but did not evaluate particulate gels with no added salt. The solution pH dictates the total charge on the protein whereas the ionic strength determines the degree of interaction among the charges (Dill, 1990) due to mobile ions shielding ionizable amino acids (Ziegler and Foegeding, 1990). For pH treatments, the net charge on the protein will regulate the attractive/repulsive forces present among proteins, which may regulate protein collisions. Hermansson (1982) describes a gel as the "right balance between protein/protein and protein/solvent interactions." At the pI, the attractive forces will be maximized and there will be an excess of protein/protein interactions, resulting in a gel with low fracture stress and strain. As the pH is increased above the pI, the protein becomes more negatively charged and the protein/solvent interactions become more balanced. The optimal balance appears to occur at pH 5.5 as indicated by Ziegler and Foegeding (1990), beyond

which a decrease in fracture stress can be seen. When aggregation is kinetically controlled, as opposed to thermodynamically controlled, a weak gel results (Egelandsdal, 1984). Vreeker et al. (1992) have found that whey proteins undergo diffusion-limited aggregation kinetics at pH 4.9 and reaction-limited aggregation kinetics at pH 5.4. Thus, when there is no repulsive barrier between proteins (at $\text{pH} \approx \text{pI}$), every particle collision results in the proteins "sticking" (kinetically-controlled aggregation). At pH 5.4, the proteins must have a specific orientation to aggregate, so not every collision results in aggregation (thermodynamically-controlled aggregation). This explains why stronger gels are formed in the reaction-limited pH regime. The >10 kPa decrease in fracture stress with little change in fracture strain indicates these two parameters are regulated by different factors. For NaCl treatments, the Debye length has been reduced to 1 nm for 0.1 M NaCl (Dickinson, 1992). Since particulate WPI gels are typically formed at >0.2 M NaCl, electrostatic theory does not explain changes in fracture rheology. Dill (1990) generalized that monovalent salts primarily interact with charges on the protein up to 0.1-0.2 M NaCl, and beyond that, charges are saturated, and NaCl concentration affects the solvent properties, which has also been indicated by Verheul and Roefs (1998b). Either a solvent quality effect or the dielectric constant of the solvent may explain the gradual decrease in fracture stress of WPI gels as NaCl is increased (Figure 3).

The degree of strain hardening differentiated the two treatments (Figure 5) even when fracture stress and strain had similar values (Table 1). To better understand the phenomenon regulating strain hardening at a molecular level, n-ethylmaleimide was added to the WPI dispersions at various concentrations. n-Ethylmaleimide (NEM) is a thiol blocker that reacts with free thiol groups (Creighton, 1993) and has been used extensively in studies to alter the gelation of globular protein gels (Shimada and Cheftel, 1988; Doi et al., 1990; Wang and

Damodaran, 1990; Matsudomi et al., 1991; Sato et al., 1995). At excessive molar ratios, it effectively inhibits gelation (Matsudomi et al., 1991), but used at a molar ratio of less than one, it has actually been shown to enhance interactions, perhaps due to increased molecular flexibility of the proteins (Xiong et al., 1993). Hoffmann and van Mil (1997) have demonstrated that up to a molar ratio one, NEM reacts preferentially with the free thiol group of β -LG, not any sulfhydryl/ disulfide exchange products. Interestingly, in this study, 2 mM NEM reduced $R_{0.3}$ to values NSD to the pH 5.47 treatment without significantly affecting fracture stress or strain (Table 1). Assuming β -LG as the main source of free thiol groups, a 10% protein WPI gel is ~ 5.5 mM protein. Thus, the molar ratio required to reduce the strain hardening is $\sim 1:3$ NEM to β -LG. This indicates that disulfide bond formation largely regulates strain hardening in WPI gels. It has been thoroughly demonstrated that increasing the pH from isoelectric to neutral conditions increases the reactivity of the thiol group (Shimada and Cheftel, 1988; Liu et al., 1994; Monahan et al., 1995). Using data from Monahan et al. (1995) and Liu et al (1994), after heating β -LG or WPI dispersions at 80°C , they calculated free sulfhydryl contents for pH 5.5 and 7.0 treatments of 31 and 37 (interpolated value) $\mu\text{moles/g}$, respectively. The free sulfhydryl value at pH 7 is $\sim 16\%$ higher than at pH 5.5, and for $R_{0.3}$, the value at 0.25 M NaCl, pH 7 is 17% higher than the pH 5.47 value. This indicates there may be a relationship between the free sulfhydryl group and strain hardening, but more definitive research is need in this area.

Recently, a phenomenological evaluation of strain hardening behavior in gelatin gels has been carried out (Bot et al., 1996; Groot et al., 1996). Groot et al (1996) experimentally verified that strain hardening in gelatin gels was best explained by two models: 1) the fractal dimension of the polymer strands; 2) the presence of both stiff rods and flexible coils (Groot

et al., 1996). At a molecular level, Bot et al (1996) interpreted these results in terms of the helical cross-links and random coil structure that comprise gelatin. As the cross-links grow in number, the strain hardening of the stress/strain curve is amplified (Bot et al., 1996). It may be the presence of at least two structural components that causes strain hardening in gels. For WPI gels, it appears that the structural elements regulating strain hardening are disulfide bonding and possibly random coil structure. Since adding 2 mM NEM reduced $R_{0.3}$ to 1.06 (Table 1), it appears that disulfide bonding provides the majority of the strain hardening effect, yet $R_{0.3}$ values never fell below 1.05 for any treatment (Figure 5), which indicates there may be some other structural component involved in this phenomenon. This corresponds nicely to the structural model for gelatin except helical cross-links in gelatin gels can be interpreted as disulfide crosslinks in WPI gels.

Permeability and SEM were complimentary methods for the evaluation of microstructure in particulate gels. As the pore size increased as observed in SEM (Figure 6 and 7), through either increasing NaCl concentration or lowering the pH, B_{gel} significantly increased in both cases. Using a first-order approximation, Bremer et al. (1989) has indicated that B_{gel} is proportional to the particle size squared. The transition region from particulate to fine-stranded gels (often referred to as "mixed gels") generally occurs at pH 6 for pH treatments (Langton and Hermansson, 1992) and ~0.2 M NaCl, pH 7 for NaCl treatments (Bowland and Foegeding, 1995). Lower B_{gel} values occur when treatment conditions favor the fine-stranded transition region. The smaller aggregate/ pore size coupled with the presence of some fine-stranded structure may account for the smaller B_{gel} .

It is apparent that fracture properties (Table 1) are unrelated to microstructure properties (Table 2) for particulate gels. One study has related microstructure to fracture

properties. Stading and Hermansson (1993) induced increasing pore sizes (as observed in SEM) in particulate β -LG gels, by forming gels at decreasing heating rates. Notches of different sizes were applied to the gels and the fracture stress was measured. Theoretically, gels fracture at their weakest structural element. When notches were applied that were smaller than the weakest structural element, no change in fracture stress was observed. The gels became notch dependent when the notch size corresponded to the gel pore size, indicating the pores are the weakest structural element (Stading et al., 1993). At pH 5.3, the aforementioned study altered fracture stress from 25 kPa (fast heating rate) to ~8 kPa (slow heating rate). This study used only a fast heating rate, and altered the fracture stress from 27 kPa (pH 5.5) to 12 kPa (pH 5.8). Apparently, the fracture stress can be manipulated with the heating rate and the pH for particulate gels and although the microstructure appears to regulate fracture stress for the former, it does not seem to have any bearing on fracture stress for the latter. Moreover, caution should be taken in comparing heating rate, time and temperature effects to solvent effects in protein gel rheology. There does not appear to be any overriding microstructural phenomena that regulates fracture stress between the pH and NaCl treatments.

When 2 mM NEM was added to the 0.25 M treatment, the $R_{0.3}$ value significantly reduced, yet the microstructures appear qualitatively similar (Figure 8). For WPI gels heated at temperatures $<75^{\circ}\text{C}$, Verheul and Roefs (1998a) have indicated that spatial structure and gel elasticity development are related to concentration of aggregated protein at the gel point and total protein in the matrix, respectively. At higher temperatures ($>75^{\circ}\text{C}$), the aggregation kinetics are different (Iametti et al., 1996; Verheul and Roefs, 1998a) and the relationship can

not be as easily determined quantitatively. Qualitatively, these results validate that there is no relationship between gel rheology and microstructure in particulate gel formation.

CONCLUSIONS

This research demonstrates that particulate WPI gels formed by lowering the pH to near the isoelectric point and adding NaCl at pH 7, have similar fracture stress and fracture strain profiles over their relevant treatment ranges. When the pH and NaCl were specifically adjusted, two pairs were formulated that were not significantly different with respect to fracture stress and fracture strain at high stress (~23 kPa) and low stress (~13 kPa). What differentiated the pairs was the degree of strain hardening, which was a treatment specific value. Gels with added NaCl at pH 7 had higher strain hardening values than gels formed at isoelectric pH. The strain hardening of the 0.25 M NaCl treatment could be reduced with the addition of 2 mM n-ethylmaleimide, to values not significantly different than the high stress pH treatment, without affecting the fracture stress and strain. This indicates that disulfide bond formation primarily regulates strain hardening in WPI and perhaps, more broadly, globular protein gels. For gel microstructure, as the permeability coefficient of the gels became smaller, the aggregates for the formulated pairs had smaller pore sizes as observed in SEM. Lower permeability values were found for treatments (pH 5.68 and 0.25 M, pH 7) approaching mixed gel conditions (pH 6 or 0.2 M NaCl, pH 7). However, no relationships between gel microstructure and fracture rheology were observed.

FIGURE 1 – GENERALIZED STRESS-STRAIN CURVE FOR PROTEIN GELS. THE SLOPE OF THE DOTTED LINES CORRESPONDS TO G_f (UPPER) AND $G_{0.3}$ (LOWER), WHERE $G_{0.3}$ REPRESENTING 30% OF THE STRAIN AT FRACTURE.

FIGURE 2 – SCHEMATIC REPRESENTATION OF THE PERMEABILITY SYSTEM WITH A REFERENCE TUBE (a) AND A GEL TUBE (b) WHERE THE SYMBOLS REPRESENT VARIABLE MEASUREMENTS FOR EQUATION 4

FIGURE 3 – FRACTURE STRESS OF HEAT-INDUCED WPI GELS (10% PROTEIN) AT INCREASING NaCl CONC. AT pH 7 (OPEN) AND pH VALUES (CLOSED)

FIGURE 4 – FRACTURE STRAIN OF HEAT-INDUCED WPI GELS (10% PROTEIN) AT INCREASING NaCl CONC. AT pH 7 (OPEN) AND pH VALUES (CLOSED)

FIGURE 5 – $R_{0.3}$ OF HEAT-INDUCED WPI GELS (10% PROTEIN) AT INCREASING NaCl CONC. AT pH 7 (OPEN) AND pH VALUES (CLOSED)

TABLE 1– FRACTURE PROPERTIES COMPARISON OF OPTIMIZED ISOPAIR TREATMENTS AND 0.25 M NaCl, pH 7 MODIFIED WITH NEM¹

Treatment	Stress (kPa) ²	Strain	Modulus (kPa) ²	Slope Ratio ³
pH 5.47	23.6 ± 2.0 ^a	1.87 ± 0.11 ^a	12.6 ± 0.7 ^a	1.05 ± 0.03 ^a
0.25 M NaCl	22.9 ± 2.9 ^a	1.85 ± 0.06 ^a	12.4 ± 1.7 ^a	1.23 ± 0.05 ^b
pH 5.68	13.3 ± 1.4 ^b	1.89 ± 0.19 ^a	7.0 ± 0.1 ^b	1.11 ± 0.03 ^c
0.6 M NaCl	12.7 ± 0.8 ^b	1.91 ± 0.03 ^a	6.7 ± 0.5 ^b	1.26 ± 0.06 ^b
2 mM NEM	25.4 ± 0.8 ^a	1.77 ± 0.07 ^a	14.4 ± 0.8 ^a	1.06 ± 0.02 ^a

¹ Values are means ± standard deviation of at least five values for three replications

² Means within column are significantly different (p<0.001)

³ Means within column are significantly different (p<0.05)

TABLE 2– STRUCTURAL PROPERTIES COMPARISON OF OPTIMIZED ISOPAIR TREATMENTS AND 0.25 N NaCl MODIFIED WITH NEM¹

Treatment	WHC (g water-held/ g protein) ²	B _{gel} (10 ⁻¹³ m ²) ³
pH 5.47	7.45 ± 0.02 ^a	3.29 ± 0.47 ^a
0.25 M NaCl	7.25 ± 0.24 ^a	0.22 ± 0.06 ^b
pH 5.68	6.56 ± 0.22 ^b	0.29 ± 0.05 ^b
0.6 M NaCl	6.25 ± 0.06 ^b	1.27 ± 0.16 ^c
2 mM NEM	NA	0.24 ± 0.04 ^b

¹ Values are means ± standard deviation of at least three values for three replications

² Means within column are significantly different (p<0.05)

³ Means within column are significantly different (p<0.001)

FIGURE 6 – LOW MAGNIFICATION SEM MICROGRAPHS FOR WPI GELS (10% PROTEIN) FORMED AT 0.25 M, pH 7 (a), AND AT 0.6 M, pH 7 (b).
BAR = 10 μ m

FIGURE 7– LOW MAGNIFICATION SEM MICROGRAPHS FOR WPI GELS (10% PROTEIN) FORMED AT pH 5.47 (a), AND AT pH 5.68 (b). BAR = 10 μm

FIGURE 8 – HIGH MAGNIFICATION SEM MICROGRAPHS FOR WPI GELS (10% PROTEIN) FORMED AT 0.25 M NaCl, pH 7 (a) AND AT 0.25 M NaCl, pH 7 WITH 2 mM NEM (b). BAR = 1 μm

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