ABSTRACT

LEKSRISOMPONG, PHANIN. How Micro-Phase Separation Explains Gelation Properties of Globular Protein Gels. (Under the direction of Professor E. Allen Foegeding).

One of the main functionalities of food proteins is the ability to form heat-induced gels. The gelation process involves protein denaturation followed by aggregation. Altering the way molecules organize and interact in the system affects microstructure, gelation properties and ultimately texture of food products. Texture is one of the most important sensory attributes determining quality and consumer acceptability of foods. Other than sensory properties, food texture has been shown to impact human health. It is well known that altering processing parameter, solvent condition, and biopolymer mixtures creates different food structures. The objective of this research was to understand the mechanisms responsible for structural formation and gelation properties of globular protein gels based on micro-phase separation consideration.

The first and second studies investigated how micro-phase separation alters the effect of heating rate on viscoelastic and fracture properties of globular protein gels. Protein solutions (pH 3.0 - 8.5) were heated using a range of heating rates (0.1 – 35 °C/min) to achieve a final temperature of 80 °C. Single or micro-phase separated solution conditions were determined by confocal laser scanning microscopy. Under single phase conditions, gels formed by faster heating had the lowest rigidity ($G'$) at 80 °C; however, a common $G'$ was achieved after holding for 4 hr at 80 °C. Fracture properties of gels prepared by fast heating were also the weakest but extending holding time after fast heating for only 5 min produced
gels as strong as gels prepared by slower heating. This suggested that the variations seen in viscoelastic and fracture properties were simply due to variations in time allowed for proteins to form a gel network. In contrast, under micro-phase separation conditions, faster heating allowed phase separated particles to be frozen in the network prior to precipitation. Thus, gels produced by faster heating had higher $G'$ values. There was no effect of heating rate on held water; supporting previous investigations that showed the gel point sets the gel microstructure and that microstructure determines water-holding properties. Overall, the effect of heating rate appears to depend on phase stability of the protein dispersion and total thermal input. In addition, minimal differences in fracture properties and held water of EWP gels at pH 4.5 versus 7.0 were observed while whey protein isolate (WPI) gels were stronger and had higher held water at pH 7.0 as compared to 4.5. This is due to a mild degree of micro-phase separation of EWP gels across the pH range whereas WPI gels only show an extreme micro-phase separation at pH values close to the isoelectric points of the two predominate proteins, $\beta$-lactoglobulin and $\alpha$-lactalbumin. Formation and physical properties of globular protein gels can be explained by degree of micro-phase separation.

In the final series of experiments, microstructure and gelation properties of EWP/polysaccharide mixed gels were investigated. Altering polysaccharide type and concentration produces a wide range of microstructures and gelation properties of EWP/polysaccharide mixed gels. The effect of polysaccharides on these properties of the mixed gels can be grouped based on the charge density of polysaccharide. In addition to
polysaccharide charge density, an understanding of micro-phase separation model was shown to be the key to predict the microstructure of mixed gels and in turn the gelation properties.

Egg white protein and WPI are used extensively as ingredients in the food industry. Despite their similarity, they were shown to respond differently to changes in pH and interactions with polysaccharides. These variations were explained based on the degree of protein micro-phase separation. This study demonstrates how the micro-phase separation model can be used to explain gel formation and gelation properties of globular protein gels.
How Micro-Phase Separation Explains Gelation Properties of Globular Protein Gels

by
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DEDICATION

This dissertation is dedicated to my parents, grandparents, and aunts.
BIOGRAPHY

Phanin Leksrisompong was born and raised in Bangkok, Thailand. She is the third daughter of Mr. Vinai and Mrs. Phatanee Leksrisompong. In 2002, she moved to the U.S.A. for higher education at the University of Kentucky and North Carolina State University. At North Carolina State University, she received an M.S. in Food Science in 2008 under the supervision of Dr. Tyre Lanier. Afterwards, Phanin received a fellowship from the American Egg Board and joined Dr. Allen Foegeding’s lab to start her Ph.D. study in 2008. Her Ph.D. work focused on gelation properties of globular protein gels.
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CHAPTER 1

LITERATURE REVIEW
Ingredients

Albumen (Egg white)

Albumen or egg white (EW) is commonly used in both household kitchens and in the food industry due to its nutrition and unique functionalities (gelling, emulsifying and foaming properties). These unique functionalities could be due to albumen being comprised of several proteins with a wide range of chemical compositions (Table 1). It can be used as an ingredient in a variety of forms including fresh, pasteurized liquid, spray dried powder or frozen (Burley and Vadehra 1989).

Proximate composition

Albumen comprises approximately 60% of the whole egg. It is made up of 4 layers: outer thin white (23%), thick white (57%), inner thin white (17%) and chalaziferous layer (3%), from shell to yolk, respectively (Burley and Vadehra 1989). Protein is a major component of the dry matter, accounting for 9.7 to 10.6% of the albumen (Burley and Vadehra 1989). Carbohydrates (mainly glucose) can be found in two forms; linked to protein (0.5% w/w) and free (0.5% w/w). Minerals, mainly Na⁺, K⁺, Cl⁻, P and S (0.5 to 0.6%), and lipids (0.03%) are also present (Li-Chan and Nakai 1989). The variation in composition is due to many factors including breed, age, environmental conditions, feed, egg size, and rate of production (Romanoff and Romanoff 1949).

The protein composition of the thick and thin layer of albumen differs only in the amount of ovomucin; thick albumen has 4 times higher ovomucin content (Kato and others 1985). The chalaziferous layer is a gelatinous layer located at the inner most layer of
albumen covering the entire egg yolk. At both ends along the vertical axis of the yolk, the layer is twisted to form a rope-like structure known as the chalazae cord. The twisted cord stretches through the thick albumen layer suspending the yolk in the center of the egg (Kato and others 1985).

**Composition of egg white proteins and their properties**

Recently, Guérin-Dubiard and others (2006) and Mann (2007) identified 78 chicken egg white proteins, the major proteins reported coincide with those previously found but 54 of these proteins have not been reported before. These new proteins are present in small quantities and have a wide range of molecular weights. Despite extensive research on egg white proteins, little is known about the minor proteins and the functional differences among polymorphisms of these proteins are unclear. The majority of proteins in EW are globular and acidic or neutral, except avidin and lysozyme (having pI of 10.0 and 10.7, respectively). All EWP are glycosylated except cystatin and the major form of lysozyme.

The 10 major proteins in albumen are reviewed in the following sections. The physicochemical properties and biological activity of these proteins are reviewed extensively by Li-Chan and Nakai (1989) and is summarized in Table 1 (Li-Chan and Nakai 1989; Weijers and others 2006).
Table 1. Composition and molecular properties of albumen (Adapted from Li-Chan and Nakai 1989; Weijers and others 2006).

<table>
<thead>
<tr>
<th>Protein</th>
<th>% (w/w)</th>
<th>pI</th>
<th>MW (kDa)</th>
<th>$T_d$ (°C)</th>
<th>Carbohydrate moiety</th>
<th>-SH</th>
<th>S-S</th>
<th>Characteristic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovalbumin</td>
<td>54</td>
<td>4.5-4.9</td>
<td>45</td>
<td>75-84</td>
<td>Yes</td>
<td>4</td>
<td>1</td>
<td>Phosphoglycoprotein, foaming and gelling; immunogenic</td>
</tr>
<tr>
<td>Ovotransferrin</td>
<td>12-13</td>
<td>6.0-6.1</td>
<td>77.7</td>
<td>61-65</td>
<td>Yes</td>
<td>-</td>
<td>15</td>
<td>Binds iron; antimicrobial</td>
</tr>
<tr>
<td>Ovomucoid</td>
<td>11</td>
<td>4.1</td>
<td>28</td>
<td>77</td>
<td>Yes</td>
<td>-</td>
<td>9</td>
<td>Trypsin inhibitor</td>
</tr>
<tr>
<td>Ovomucin</td>
<td>1.5-3.5</td>
<td>4.5-5.0</td>
<td>0.23–8.3x10³</td>
<td>77</td>
<td>Yes</td>
<td>-</td>
<td>9</td>
<td>Viscous; role in age-thinning; viral hemagglutination inhibitor</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>3.4-3.5</td>
<td>10.7</td>
<td>14.3-14.6</td>
<td>69-77</td>
<td>No</td>
<td>4</td>
<td>4</td>
<td>Lysis of bacterial cell wall; antimicrobial</td>
</tr>
<tr>
<td>G2 ovoglutulin</td>
<td>1.0</td>
<td>4.9-5.5</td>
<td>47-49</td>
<td></td>
<td>Yes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G3 ovoglutulin</td>
<td>1.0</td>
<td>4.8, 5.8</td>
<td>49-50</td>
<td></td>
<td>Yes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ovoinhibitor</td>
<td>0.1-1.5</td>
<td>5.1</td>
<td>49</td>
<td></td>
<td>Yes</td>
<td></td>
<td></td>
<td>Serine proteinase inhibitor; wide spectrum of inhibitory activity</td>
</tr>
<tr>
<td>Ovoflavoprotein</td>
<td>0.8</td>
<td>4.0</td>
<td>32-36, 80</td>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td>Binds riboflavin</td>
</tr>
<tr>
<td>Ovomacroprotein</td>
<td>0.5</td>
<td>4.5-4.7</td>
<td>760-900</td>
<td></td>
<td>Yes</td>
<td></td>
<td></td>
<td>Strongly antigenic; high immunological cross-reactivity</td>
</tr>
<tr>
<td>Cystain</td>
<td>0.05</td>
<td>5.1</td>
<td>12</td>
<td></td>
<td>No</td>
<td></td>
<td>1</td>
<td>Thiol proteinase inhibitor</td>
</tr>
<tr>
<td>Avidin</td>
<td>0.05</td>
<td>10</td>
<td>55-68.3</td>
<td></td>
<td>Yes</td>
<td></td>
<td>1</td>
<td>Binds biotin; antimicrobial</td>
</tr>
</tbody>
</table>
**Ovalbumin**

Ovalbumin is a major protein in albumen, making up 54% (w/w) of total EWP (Li-Chan and Nakai 1989). It is a phosphoglycoprotein consisting of approximately 3.2% (w/w) carbohydrates (Fothergill 1970) and phosphate moieties attached to the polypeptide chain. The purified ovalbumin is made up of three components differing in phosphorus content. It has a compact spherical shape, having a molecular weight (MW) of 45 kDa (Fothergill and Fothergill 1970) and the carbohydrate moiety has a MW of about 1.58 kDa. It is composed of 385 amino acids (Nisbet and others 1981) with one-third of the amino acids being charged and half of its amino acids are hydrophobic (Li-Chan and Nakai 1989). Ovalbumin has pI around 4.6 and is negatively charged at physiologic pH.

Ovalbumin has significant cystine content, 2.17% (w/w). There are six buried Cys residues in an ovalbumin polypeptide chain; four thiol (free sulfhydryl) groups and one solvent-accessible disulfide bridge (Cys 73 - Cys 120) (Fothergill and Fothergill 1970; Nisbet and others 1981; Mine 1995). Ovalbumin is the only protein in albumen consisting of free sulfhydryl groups capable of inducing sulfhydryl-disulfide interchange reactions upon changes in storage conditions, pH, and denaturation (Fothergill and Fothergill 1970; Lechevalier and others 2003).

Thermal aggregation and gelation properties of ovalbumin have been studied extensively (Doi and Kitabatake 1989; Arntfield and others 1990; Harte and others 1992; Kitabatake and Kinekawa 1995; van der Linden and Sagis 2001; Weijers and others 2002). Ovalbumin has a denaturation temperature ($T_d$) of 84 °C (Donovan and Mapes 1976). Above critical concentration for gelation, ovalbumin forms transparent gels at high pH (above 7.0)
and low ionic strength (Kitabatake and Kinekawa 1995). Moreover, ovalbumin can be isolated by anion-exchange or crystallization method (Johnson and Zabik 1981; Croguennec and others 2000; Kosters and others 2003).

**S-ovalbumin**

During storage at high temperature and pH (9.0), ovalbumin has the ability to be irreversibly converted to a more thermally stable form known as “S-ovalbumin” (Smith and Back 1962, 1965; Takahashi and others 1996; Sugimoto and others 1999; Arii and others 1999) having a $T_d$ of 92.5 °C (Donovan and Mapes 1976). The amount of S-ovalbumin depends on the storage time and pH of the eggs. Therefore, ovalbumin behaves as a mixture of two proteins; ovalbumin and S-ovalbumin. There is no difference in amino acid composition shown between these two forms of ovalbumin. Previously, it was proposed that a sulfhydryl-disulfide interchange might be responsible for the difference in the properties (Smith and Back 1962 1965). However, a comparison of the soluble peptides obtained from tryptic digestion did not show any significant changes in the peptides linked by this bond (Smith and Back 1968). S-ovalbumin has a pI lower than ovalbumin (Schafer and others 1999) and it is shown to be more compact, explaining why it is more heat stable (Takahashi and others 2005). From the commercial point of view, this conversion is important since S-ovalbumin is highly resistant to thermal aggregation which makes egg a useful ingredient in some applications (Smith and Back 1968).
Conalbumin (Ovotransferrin)

Conalbumin is the second most abundant protein in EW, comprising approximately 12 to 13% (w/w) of the total protein content. Both ovalbumin and conalbumin can form stronger gels and gel at lower concentrations than low MW polymers (Tang 1994). Conalbumin is a large linear protein having MW of 76 kDa and a pI around 6.0. It is the most heat-labile protein, having the lowest $T_d$ (65 °C) of all EWP at pH 7.0, suggesting that conalbumin will aggregate quickly (Matsudomi and others 2002). When compared to ovalbumin, conalbumin is more sensitive to heat but less susceptible to surface denaturation. Unlike ovalbumin, conalbumin does not contain phosphorus or free sulfhydryl groups. However, conalbumin contains 15 disulphide bonds, therefore it is likely to interact with proteins containing sulphydryls by sulphhydryl-disulfide exchange. Moreover, it consists of 2 lobes, each containing a specific binding site for metal ions such as iron, copper, zinc, or aluminum. The biological functions of conalbumin have been recognized as an iron binding glycoprotein that inhibits the action of trypsin as well as inhibiting bacterial growth (Li-Chan and Nakai 1989; Jeffrey and others 1998). Binding of metal ions significantly increases heat stability of conalbumin.

Ovomucoid

Ovomucoid is the primary allergenic protein in egg (Cooke and Sampson 1997) and is antigenic, even after boiling (Urisu and others 1997). It makes up approximately 11% (w/w) of albumen. It is well known for inhibiting trypsin; having three tandem domains similar to pancreatic secretory trypsin inhibitor (Kazel-type). Ovomucoid has nine disulfides
but no free sulfhydryl groups and a pI of 4.1. It is a water-soluble, highly glycosylated protein having carbohydrate attached to the polypeptide at asparaginyl residues (Yamashita and others 1982). It consists of three separate domains each cross-linked by 3 disulfide bonds. Ovomucoid has a MW of 28 kDa where 25 to 30% (w/w) is carbohydrate (Yamashita and others 1982) consisting of D-mannose, D-galactose, glucosamine, and sialic acid (Matsuda and others 1982). It is very heat stable in acidic and mild alkaline solutions and proposed to assist in stabilization of thermal transitions during heating. The $T_d$ of ovomocoid is very pH dependent (Matsuda and others 1982).

**Ovomucin**

Ovomucin makes up 1.5 to 3.5% (w/w) of albumen. Ovomucin has three subunits ($\alpha_1$-, $\alpha_2$-, and $\beta$-ovomucin), that are different in molecular mass, solubility, carbohydrate content, and composition (Robinson and Monsey 1975; Hayakawa and others 1983; Itoh and others 1987; Watanabe and others 2004). It is a sulfated glycoprotein having MW of 0.23 to $8.3 \times 10^3$ kDa. The carbohydrates are bound by disulfide bonds (Itoh and others 1987) and make up approximately 33% of the total MW, giving ovomucin the highest viscosity out of all egg white proteins (Kato and others 1985; Johnson and Zabik 1981; Li-Chan and Nakai 1989).

Haugh unit (HU) measures albumen height and is an indicator of egg quality (i.e., freshness). When ovomucin was isolated from thick albumen of fresh eggs with high HU, the yield of ovomucin was four times higher than the amount isolated from older eggs with low HU. In contrast, the total amount of ovomucin from thin albumen of eggs with high and
low HU did not differ (Toussant and Latshaw 1999). Ovomucin has low solubility (Rabouille and 1990) and a pI at a slightly acidic pH of 4.5 to 5.0. The phenomenon of egg white thinning during storage is well known. One of the mechanisms proposed for thinning is the dissociation of ovomucin-lysozyme complex as the pH increases over time. In addition, a decrease of thick albumen and an increase in free sulfhydryl groups in the albumen result in a loss of foam volume and gel hardness (Wong and Kitts 2003).

**Ovoglobulins, (Lysozyme (G₁), G₂, and G₃)**

The three ovoglobulins were discovered in 1940 by Longsworth and co-workers. Later on G₁ was characterized and called lysozyme which is an enzyme known for its ability to lyses cell walls of bacteria. It is a strongly basic protein having pI at 10.7, which is much higher than other egg white proteins. It is positively charged at a neutral pH and can interact with other negatively charged proteins. It has the lowest MW (14.3 kDa) comprised of 129 amino acid residues with no carbohydrate moiety. The structure is stabilized by 4 disulfide bridges, giving it rigid structure but no free SH groups (Li-Chan and Nakai 1989). Heat inactivation of lysozyme is pH and temperature dependent. When lysozyme is in the freshly laid eggs (~ pH 7.0), it is more heat sensitive than when it is present in a higher pH. Ovoglobulin G2 and G3 are similar, both comprising approximately 4% (w/w) of albumen (Li-Chan and Nakai 1989) and have MW of 36 and 45 kDa, respectively. They are good foaming agents and play an important role in foaming properties of EWP (Sugino and others 1997).
**Ovoinhibitor**

Apart from ovomucoid, ovoinhibitor is another trypsin inhibitor found in albumen. Ovoinhibitor is a multihead inhibitor which inhibits bacterial serine proteinase, fungal serine proteinase, and chymotrypsin (Li-Chan and Nakai 1989).

**Ovomacrogllobulin**

Ovomacrogllobulin is the second largest egg glycoprotein after ovomucin and its MW is 760-900 kDa. Ovomacrogllobulin, like ovomucin, has the ability to inhibit hemagglutination (Sugino and others 1997).

**Ovoflavoprotein**

Flavoprotein is an apoprotein binding to riboflavin (vitamin B) at a one to one ratio. It has a MW of 32 to 36 kDa. Its pI is 4.2, above and below this pH, the ability to bind riboflavin is lost (Sugino and others 1997).

**Avidin**

Avidin has been intensively studied, even though it only comprises 0.05% (w/w) of the total protein in albumen, because it can tightly bind biotin (vitamin B) (Eakin and others 1941). Avidin is a strongly basic glycoprotein having a pI of 10. Avidin is a stable tetramer composed of four identical monomers having a total MW of 68.3 kDa. Each avidin subunit binds to one molecule of biotin (Green and Toms 1973). The high affinity constant of avidin for biotin has been widely used in molecular biology, affinity chromatography, molecular
recognition and labeling, Enzyme Linked Immuno Sorbent Assay (ELISA), histochemistry, and cytochemistry (Wilchek and Bayer 1990).

**Ovoglycoprotein**

Ovoglycoprotein is an acidic glycoprotein with a MW of 24.4 kDa. This protein contains hexoses (13.6%), glucosamine (13.8%) and N-acetyleneuraminic acid (3%) (Ketterer 1965). The biological functions of ovoglycoprotein are still unclear.

**Whey Protein Isolate**

Whey is a by-product of cheese and casein manufacturing; the watery fraction of milk released from the curd. It is composed of lactose, proteins, inorganic salts, vitamins and a small amount of other components (Fox 2003). “Sweet whey” refers to liquid remaining after enzymatic precipitation and “acid whey” refers to liquid remaining after isoelectric precipitation of casein from milk. Historically, whey has been considered a waste product and was discarded. For the past ~ 45 years however, whey protein (WP) has become known for its high nutritional value and used in functional applications such as gelling, emulsifying, and foaming, making it a popular ingredient in manufactured food products (Swaisgood 1982; Kinsella and Whitehead 1989; Wong and others 1996; Foegeding and others 2002; Fox 2003; Kilara and Vaghela 2004).

The concentration of protein in whey is approximately 0.6% (w/v) and isolation processes are used to make high protein ingredients. Protein concentration can be increased by a variety of approaches including ultrafiltration (Swaisgood 1996; Wong and others
Ultrafiltration, the most commonly used method, concentrates proteins in the retentate and removes low MW molecules (lactose and minerals) and water in the permeate. These processing technologies have made it economically possible to concentrate and exploit WP as a food ingredient (Morr and Foegeding 1990). Currently, there are different types of WP ingredients sold commercially, however the two main types are whey protein concentrate (WPC) and whey protein isolate (WPI) (Morr and Foegeding 1990), having protein content of 25 to 90% and greater than 90% protein, respectively.

Whey protein accounts for approximately 20% of the total protein found in milk. The protein fractions of whey are comprised of a number of individual proteins including β-lactoglobulin (β-lg), α-lactalbumin (α-la), bovine serum albumen (BSA), and immunoglobulins (Swaisgood 1982). Out of all the proteins, β-lg and α-la are present in the highest concentrations and thus contribute the most to the functionality of WP ingredients. The amino acid composition, sequences, and structural characteristics of these proteins are well characterized (Kinsella and Whitehead 1989; Swaisgood 1996). A summary of composition of whey protein is shown in Table 2.

**β-lactoglobulin (β-lg)**

β-lactoglobulin is a major component, approximately 70%, of WP (Swaisgood 1982). It is one of the most intensively studied globular proteins. It was first isolated in 1934 by Palmer and was first known as “β-lactalbumin”. Its name was changed over time to “β-lactoglobulin” to reflect its globulin characteristics. It is insoluble in pure water at the
isoelectric point (pH 5.2) but can be dissolved in dilute salt solutions, however, it also has albumin characteristic (Fox 2003). β-lactoglobulin is comprised of 162 amino acids and exists in more than 20 different genetic variations (Sawyer 2003). The most predominant forms are β-lg A (18,362 Da) and β-lg B (18,276 Da), presenting at almost equal frequencies (Wong and others 1996). β- lactoglobulin A and B differ by two amino acids; Asp$_{64}$ and Val$_{118}$ in β-lg A is replaced by Gly$_{64}$ and Ala$_{118}$ in β-lg B, resulting in different structural characteristics (Qin and others 1999).

β-lactoglobulin has a diameter of about 2 nm and consists of 9 anti-parallel β-sheet strands. X-ray crystallography shows that eight of the strands exist in the core of the barrel (strands A-H) and one strand (strand I) exists on the outside which is able to form part of the dimer interface. The opening is lined with hydrophilic amino acids while the interior is high in hydrophobic amino acids (Papiz and others 1986). In the pH range of 3.5 to 5.2, β-lg A can form octamers (MW~144kDa) (Walstra and Jenness 1984). As the pH increases to around 7.5, β-lg forms dimmers through non-covalent interactions (Pessen and others 1985). At pH lower than 3.5, or greater than 7.5, the dimer dissociates into monomers as the net charge increases. At pH greater than 9.0, β-lg undergoes irreversible denaturation (Wong and others 1996). β-lactoglobulin exhibits an extreme solubility at pH 2.0, where it is strongly ionized (21 positive charges for 162 amino acids). The monomers of β-lg contain two disulfide bonds (Cys$_{66}$ - Cys$_{160}$ and Cys$_{106}$ - Cys$_{119}$) and one free thiol group (Cys$_{121}$) (Papiz and others 1986). At above pH 7.5, the thiol group becomes reactive (Tanford and others 1959; Uhrinova and others 2000) which can participate in thiol disulfide interactions (Kinsella and Whitehead 1989).
Table 2. Composition and molecular properties of whey protein (Adapted from Swaisgood 1982).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Concentration (g/L)</th>
<th>Molecular weight kDa</th>
<th>Isoelectric point</th>
<th>Important characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whey protein</td>
<td>5-7</td>
<td></td>
<td></td>
<td>Dimer; Compact globular structure; 2-S-S- and 1 –SH</td>
</tr>
<tr>
<td>β-lactoglobulin</td>
<td>2-4</td>
<td>18</td>
<td>5.2</td>
<td></td>
</tr>
<tr>
<td>α-lactalbumin</td>
<td>1-1.5</td>
<td>14</td>
<td>4.8</td>
<td>Naturally binds one Ca2+; 4 –S-S- and no –SH</td>
</tr>
<tr>
<td>Serum Albumin (BSA)</td>
<td>0.1-0.4</td>
<td>66</td>
<td></td>
<td>A highly structured but flexible protein; 17 –S-S- and 1 –SH</td>
</tr>
<tr>
<td>Immunoglobulins</td>
<td>0.6-1</td>
<td></td>
<td></td>
<td>Antibodies</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>Variable for cow</td>
<td>80</td>
<td>8.7</td>
<td>Iron-binding proteins; Red colored protein</td>
</tr>
<tr>
<td>Proteose-peptone</td>
<td>0.6-1.8</td>
<td></td>
<td></td>
<td>Derived from caseins by proteolysis; a number of minor proteins which are indigenous to milk</td>
</tr>
<tr>
<td>Indigenous enzymes</td>
<td>Minor</td>
<td></td>
<td></td>
<td>Lipase, proteinases, phosphatases, nuclease, lactoperoxidase</td>
</tr>
</tbody>
</table>
The quaternary structure of β-lg is sensitive to both heat and pH. Upon heating, the β-lg dimer first dissociates to monomers at 30 to 55 °C (Sawyer 1969). Above 65 °C, β-lg starts to unfold, exposing highly reactive hydrophobic groups that increases hydrophobic interactions between molecules, resulting in aggregation (Kinsella and Whitehead 1989). Thiol reactivity also increases (Larson and Jenness 1952) resulting in disulfide interchange and aggregation (Sawyer 2003). Heat denaturation studies using differential scanning calorimetry show that pH, ionic strength, temperature, and protein concentration determine the denaturation temperature \(T_d\) of β-lg (de Wit and Klarenbeek 1981). The protein is most stable at pH 6.0 and most sensitive at pH 4.0 (Wong and others 1996). Addition of sugars has a stabilizing effect on heat denaturation of β-lg (Park and Lund 1984; Boye and Alli 2000; Anema and others 2006).

**α-lactalbumin (α-la)**

α-lactalbumin is the second most prevalent protein in whey, comprising approximately 25% of WP (Kinsella 1989). It is a compact globular protein composed of 162 amino acids having a MW of 14 kDa (Brew and Grobler 1992). The molecule has an ellipsoid shape with two distinct lobes divided by a cleft. One lobe is comprised of four helices and the other is comprised of two β-strands with a loop-like chain (Wong and others 1996; Acharya and others 1989). α- lactalbumin has four intramolecular disulfide bonds but no free thiol groups, which limits its conformational flexibility in some solvent conditions (Brew and Grobler 1992). It has a structure similar to lysozyme (Nitta and Sugai 1989); 54 of 123 residues in α-la primary structure are identical to lysozyme and four disulphide bonds
have similar locations in the main chain (Swaisgood 1982; Walstra and Jenness 1984; Brew 2003). At pH 7.0, $T_d$ of $\alpha$-la is 63.7 °C (McGuffey and others 2005). Similar to β-lactoglobulin, addition of sugar will stabilize $\alpha$-la against thermal denaturation (Boye and Alli 2000; Anema and others 2006).

**Other Protein Components**

Bovine serum albumin is another major protein found in whey, but in a very small amount (0.3 to 1%) compared to β-lg and $\alpha$-la. It is composed of 582 amino acids (Fox 2003) and has a MW of 69 kDa (Suttiprasit and others 1992) and a pI around 4.7. It is a globular protein with an elliptical shape (Fox and McSweeney 1998) containing 17 disulfide bonds and 1 free sulfhydryl group (Fox 2003). Other protein components in whey include proteose-peptone, immunoglobulins, lactoferrin, and indigenous enzymes. Bovine serum albumin and other minor whey proteins made up approximately 5% of whey proteins. When using proteins from different sources, the existence and variation of these components should be taken into consideration.

**Polysaccharides**

The extensive practical significance of polysaccharides in many food products make them valuable and commonly used as food ingredients. Polysaccharides are used as thickening, stabilizing, and gelling agents to increase the shelf life and stability of foods and also used to create different textures of food products. Unlike proteins, many polysaccharide solutions gel upon lowering temperature after heating and/or with addition of specific cations.
(Morris and others 1998). There are many polysaccharides available in the market and the number is increasing. This literature review focuses on three type of polysaccharides; galactomannans, pectins and carrageenans.

**Galactomannans**

Many *Leguminosae* contain galactomannans, which are polysaccharide reserves in plants existing in the seeds endosperm (Dierckx and Dewettinck 2002). The commonly known galactomannans are locust bean gum (LBG) and guar gum which are mainly used as thickeners in food. Locust bean gum or *Ceratonia siliqua* originated from the Middle East. The fruit is about 10 to 20 cm in length and contains approximately 10 to 15 seeds, known as carob beans, which are the source of the polysaccharide. Guar gum originally from India and Pakistan, is obtained from the seeds of *Cyamopsis tetragonolobus*. It has been commonly used in both feed and food in these countries.

Galactomannans are linear polysaccharides comprised of mannose units linked by 1,4-β-D-glycosidic bonds at which the hydrogen atom of several primary hydroxyl groups on C-6 are substituted by single α-D-galactose units by 1, 6 linkages (Wielinga and Maehall 2000) (Fig 1).

![Figure 1a. Structure of guar gum (de Jong and van de Velde 2007).](image)
The ratio of galactose-mannose ranges from 1 to 10 depending on the type and source (Daas and others 2000). Guar gum and LBG have average galactose to mannose ratios of 1:2 and 1:4, respectively. The average galactose to mannose ratio is the main difference between guar gum and LBG, giving them different properties. Guar gum is more soluble in water than LBG (Dierckx and Dewettinck 2002). The ratio has been reported to effect the solubility of the galactomannans, substitution of the mannan chain by galactose makes the galactomannans more water soluble (Alistair 1995). Moreover, these gums show a wide molecular weight polydispersity, even when obtained from the same source (Doublier and Launay 1981; da Silva and Goncalves 1990).

**Pectins**

Pectins have been used in food since the first fruit preserves were made and were first isolated in 1825 (May 2000). Pectins are found in most land plants, mainly in soft tissue of shoots, leaves and fruits. Despite the abundance, not all pectins can be used commercially. Pectins are a partial breakdown product of complex structures in the plant cell wall making them heterogeneous in nature. Unlike microbial polysaccharides, it is not known if pectins
have a definite oligosaccharide repeat units. Commercially, pectins are extracted mainly from lemon peel, lime, orange peel and apple pomace (May 2000). The extracted polymers are linear and relatively stiff (Morris and others 1982). Known as homogalacturonans, they consist mainly of a 1, 4 linked α-D-galacturonate (typically ~90%) to which neutral sugars are attached (<10%).

![Structure of pectin](image.png)

Figure 2. Structure of pectin (de Jong and van de Velde 2007).

Pectins are acidic polysaccharides with carboxylic groups bearing the negative charge and partially esterified with a methoxyl group on the C-6 (Willats and others 2001). Mainly there are two types of pectins available commercially which are categorized based on the degree of esterification (DE) or also known as degree of methoxylation (DM). Typically, high methoxyl pectin has 70% DE and low methoxyl pectin has 35% DE. Degree of esterification dictates the charge density of the polysaccharide corresponding to 0.3 and 0.65 mol/mol of monosaccharide for high methoxyl (HM) and low methoxyl (LM) pectin, respectively. Degree of esterification and their distribution as well as molecular weight dictate the behavior of pectins. Pectins are not the most viscous polysaccharides, therefore they are not commonly used to increase viscosity. However, they are sometimes used to replace sugar in drinks where a small increase in viscosity is desired to give mouth feel.
Pectins can form complexes with positively charged polymers when mixed in solutions. This type of interaction is known as complex coacervate (Glahn 1982), which is the mechanism stabilizing the system of acid milk and soy milk between pectins and proteins. Pectins are found in many products ranging from low sugar jam, confectionery jellies, and processed cheese.

**Gelation mechanism**

High methoxyl and LM pectin have different gelation mechanisms. Pectin is known for its gelling ability but will only gel under specific conditions. For HM pectin, sugar concentration and pH influence the optimum condition; gel networks form under acidic conditions and high solid content. The pectin chains must be partially dehydrated and the negative charge must be low enough to allow chain-chain interactions. On the other hand, LM pectin gels are formed by networks induced according to the egg box model proposed by Powell and others (1982). The gelation of LM pectins is a two-step process; the first step being formation of dimeric junction zones of roughly consecutive 12-16 galacturonic acid units (Kohn and Luknar 1975; Powell and others 1982) and the second step is the aggregation of dimmers with the presence of soluble calcium salt (which is sometimes found naturally in milk or fruit).

**Carrageenans**

Carrageenans are naturally occurring polysaccharides extracted from the cell wall of cold and warm water red seaweeds, *Chondrus crispus* and *Eucheuma*, respectively.
Carrageenans are extensively used in the food industry as thickening and gelling agents since they can be used to create a wide range of textures and stabilize dairy products (high milk protein reactivity) (Imeson 2000). Carrageenans are linear polysaccharides comprised of repeating galactose backbone and 3, 6–anhydrogalactose linked by alternating \( \alpha\)-(1,3) and \( \beta\)-(1,4) glycosidic bonds (Fig 3) (Rees 1963). There are three main types of carrageenan; \( \kappa\)-, \( \iota\)-, and \( \lambda\)-carrageenan which differ in their degree of sulfation. \( \kappa\)-, \( \iota\)-, and \( \lambda\)-carrageenan contain approximately 25, 32, 35% ester sulfate, respectively (Rees 1969; Knutsen and others 1994).

![Figure 3a. Structure of \( \kappa\)-carrageenan (de Jong and van de Velde 2007).](image)

![Figure 3b. Structure of \( \iota\)-carrageenan (de Jong and van de Velde 2007).](image)

**Gelation mechanism**

At high temperatures, carrageenans are hydrated and exist as random coils due to thermal agitation. Gelation occurs upon cooling and can be viewed as a two-step process.
The first step being transition from a disordered coil to an ordered helix state, followed by the second step of aggregation and formation of junction zones between individual helices (Fig 4). Most κ- and ι- carrageenan solutions will set into a gel structure between 40 and 60 °C. The gelling behavior is strongly influenced by the nature and concentration of cations present in the solution as well as by the polymer concentration and cooling rate. κ-carrageenan requires potassium ions to form strong and rigid gels with some syneresis (Fig 5A). Whereas addition of calcium is required for ι-carrageenan to form elastic gels with good water holding properties and freeze/thaw stability (Fig 5B) (Imeson 2000). A blend between κ- and ι- carrageenan has been shown to give unique freeze/thaw stability properties to mixed gels, with ι-carrageenan imparting the freeze/thaw stability to κ-carrageenan gels (Christensen and Trudsoe 1980). Carrageenans show thermoplastic behavior since they melt upon heating and form a gel upon cooling. Carrageenans are not stable in acidic conditions; auto hydrolysis occurs at pH values below 4.3 which causes the viscosity and gel strength to weaken (Hoffmann and others 1996).

Figure 4. Gelation mechanism of κ-carrageenan (Morris and others 1998).
Rheology

Rheology is the study of deformation and flow of matter (Macosko 1994). When designing new food products, it is important to understand their rheological properties since these behaviors can vary dramatically even within the same product type when processing conditions change (Steffe 1996). Through rheology, the right formulations and processing conditions can be determined to optimize food quality (Daubert and Foegeding 1998; Zhong and Daubert 2007).

Based on rheological and sensorial properties, van Vliet and others (2009) classified food materials as liquids, semi-solids, soft-solids and hard-solids. Although, both soft- and hard-solids require chewing to reduce particle size, soft-solids do not have the crispy attributes associated with hard solids. This review of literature will only focus on soft-solid foods. The examples of soft-solids are gel based food products including sausages, surimi, cheese, and hardboiled egg white.
Viscoelastic material

Soft-solid materials are comprised of two or more components; one of which is liquid present in a large amount. These materials exhibit viscoelastic behavior; having characteristics of elastic solids and viscous fluids, depending on the applied conditions. In an ideal elastic system, the stress and strain relationship is linear up to the point of fracture and independent of speed. Polyacrylamide gels have been used as a model system for a soft-solid, elastic material (Foegeding and others 1994). However, elastic behavior alone does not describe the behavior of soft-solid foods since viscoelastic materials show ductile behavior which has linear stress strain relationship before moving into a non-linear region before fracture. These materials show time dependent behavior; they respond differently to different strain rates (speed of application) and time of process. Therefore, it is important to understand the importance of the deformation time scale.

\[ De = \frac{t}{T} \]  

Equation 1

The Deborah number (De) is a ratio between the characteristic response time of a material (t) to the characteristic time scale of an experiment (T) (equation 1). The smaller the number (De \( \ll \) 1), the more fluid-like behavior the material will have and vice versa (Steffe 1996).
The force (stress) verses deformation (strain) curve of viscoelastic gels is usually composed of three main regions: linear, non-linear, and fracture regions (Fig 6). Probing the materials in all regions gives a better understanding of their rheological properties (Barrangou and others 2006; Foegeding 2006). There are many methods that can be used to determine the rheological properties of soft-solid gels in these regions.

**Linear region (small-strain deformation)**

Small strain measurement is a non-destructive method used to investigate gel network formation in the linear region. Devices such as small amplitude oscillatory shear (SAOS) (Beveridge and others 1984) or transient experiments (i.e., creep compliance or stress relaxation) can be used (Katsuta and others 1990). The information obtained in the liner region is important for understanding time-dependent viscoelastic properties (Ross-Murphy 1995). Unlike measuring fluids where steady shear rate is used, dynamic shear is used to
understand elastic and viscous properties of the viscoelastic materials. In SAOS tests, the material is subjected to dynamic shear where sinusoidal stress (stress-controlled) is applied to the material and the response signals of dynamic modulus ($G^*$) and $\tan \delta$ are monitored.

\[ \tan \delta = \frac{G''}{G'} \]  

Equation 2

Storage modulus ($G'$) is the elastic component which reflects the energy stored in the network. Conversely, loss modulus ($G''$) is the viscous component reflecting energy dissipated. Different materials respond to the sinusoidal stress differently. Hookian solids are pure elastic and their strain will fall in-phase with applied stress. Whereas, Newtonian liquid is ideally viscous and its strain is 90 degree out-of-phase with applied stress (Hamann 1983).

Small amplitude oscillatory shear is an important method used to study gelation phenomenon. It can be used to monitored real time information on the effects of environmental conditions (i.e., time and temperature) on the gelation processes and gel properties at a fixed frequency (Bot and others 1996). Moreover, SAOS is a more accurate method used to detect the gel point temperature at specific frequencies.

**Mechanical spectra**

The SAOS measures the moduli at a single frequency. However, a good understanding of the frequency (i.e., time) dependence of viscoelastic materials is essential (Zhong and Daubert 2007). A full rheological characterization on the fully cured system can
be done by performing frequency sweeps. This method is performed at a fixed strain within the linear viscoelastic region (LVR) to characterize the mechanical spectra of material by oscillating the material in a wide range of frequencies (usually within 0.01 and 100 radians/s). Mechanical spectra also allows for distinction among physical states: gels, concentrated solution, and dilute solutions (Fig 7). Many studies of food systems often perform in the LVR to understand the basic physical properties of viscoelastic solids.

Figure 7. Mechanical spectra of three types of viscoelastic material (Adapted from Zhong and Daubert 2007).

Non-linear region

The non-linear region is a pre-fracture region which falls in between LVR (typically strain less than 0.1) and the fracture region. Some materials show strain hardening (increase in stress as the strain increases), and others show strain weakening (decrease in stress as the strain increases) properties. Sharda and Tschoegl (1974) and Peleg (1984) developed models that deviate from large-strain/ideal elastic behavior in order to understand the non-linearity. The BST equation (Blatz and others 1974) has been applied most recently to biopolymer gels to understand their non-linear behavior, however, interpretation of the parameters estimated from this model has varied (McEvoy and others 1985; Bot and others 1996; Groot and others 1996).
Large strain (Fracture properties)

Large-strain deformation is used to investigate the fracture properties of fully cured systems in the non-linear region. Both strong and weak gels may have similar mechanical spectrum; $G'$ is greater than $G''$ across frequencies in the small strain region (Fig 7), however they have very different fracture properties. The study of large deformation behavior of food has increased in the past years since fracture properties are more related to sensory texture perceived by humans and product stability during handling than small strain rheological properties (Montejano and others 1985).

Fracture (i.e., mechanical breakdown) properties of gels are related to weaknesses in the structure due to defects and/or inhomogeneous elements in the network (i.e., cracks, pores, or weak regions) (McEvoy and others 1985; Luyten and others 1992). Upon deformation, fracture starts at the defect surface where the local stress is higher than the overall stress of the material (Walstra 2003). According to van Vliet and Walstra (1995), materials fracture when “bonds between the structural elements of a material in a certain macroscopic plane break, resulting in a breakdown of the structure of the material over the length scales much larger than the structural elements, and ultimately a falling apart of the material”.

Different fundamental methods have been developed to investigate fracture properties as well as to correlate these properties to the sensory texture. These methods include twisting, compressing, or extending the samples up to the fracture point after which the samples are permanently destroyed (Hamann 1983; Hamann and MacDonald 1992). Out of all these methods, uniaxial compression is the most commonly used because sample
preparation is simple and less time consuming. However, this technique is considered to be more complicated than others since the stresses can be distributed through the material in a variety of directions. Moreover, the sample deformation and release of water during compression must be understood to account for changes in the cross-sectional area during deformation (van den Berg and others 2007). In torsional deformation, a capstan shaped sample is twisted to the point of fracture to determine the fundamental parameters of fracture stress ($\sigma_f$) and fracture strain ($\gamma_f$). The materials will fracture in their weakest mode since shear force, compressive, and tensile stresses of equal magnitude are created (Diehl and Hamann 1979; Diehl and others 1979). Deformation occurs in pure shear, thus the samples that exude fluids in uniaxial compression exude little or no fluid in torsion, as shape changes are minimized (Hamann and MacDonald 1992). Empirical methods, such as the penetration test and Texture Profile Analysis (TPA), are also used frequently to determine the mechanical properties of gels. However, these methods cannot correlate the chemical interactions and physical structures responsible for texture formation because of their empirical nature (Foegeding 2007).

### Protein gelation

Gelation of protein has long been a subject of interest for both academia and industries such as food, pharmaceutical, and material sciences (van der Linden and Foegeding 2009). There are many different physical and chemical means to induce protein gelation, including heat, pressure, ion, chaotropie agents (i.e., urea), acids and bases. However, this review of literature only focused on heat-induced gelation of globular proteins.
In food science, heat-induced gelation is one of the most studied phenomenon because it is responsible for the texture of gel based food products such as sausages, surimi, cheese, yogurt, and cooked egg white. A good understanding of protein gelation allows food scientists to engineer desired textures and specific functionalities as well as to prevent aggregation and gelation when undesired (i.e., protein gelation during pasteurization and sterilization). Despite its importance, the gelation mechanism of heat induced globular protein is not yet well understood because such gels exhibit very diverse microstructures and rheological properties due to their viscoelastic nature. Models are often used as one of the ways to understand and explain the gelation process.

**Early gelation model**

One of the first heat-induced gelation models was proposed by Ferry in 1948. This model illustrates that gelation is a two-step process; the first step being denaturation and exposure of active sites for inter-molecular interactions and the second step being aggregation and interactions along the entire molecules to form a three dimensional network (Fig 8). This model implies that a critical structural change (unfold) is key for proteins to aggregate.

![Figure 8. Heat-induced gelation model of protein (Ferry 1948)](image)
According to Ferry (1948), denaturation rate ($k_1$) and aggregation rate ($k_2$) determined gel microstructure type (i.e., coarse versus fine-stranded), which in turn determined macroscopic properties (appearance and water holding properties) of the gels. It is important for $k_2$ to remain slower than $k_1$ for a heat-induced gel to properly form. If $k_2$ is much greater than $k_1$, disorganized protein clusters (a coagulum) would form and the gel matrix will not be able to hold water, resulting in syneresis. If $k_2$ is slower than $k_1$, a “fine” structure gel network is formed due to more accumulation of free, long chained denatured proteins. The higher the concentration of these long chain molecules, the finer the gel network and the higher the water holding properties. Fine stranded gels are formed when the attractive forces are smaller than repulsive forces. However, a high domination of repulsive forces could result in much greater $k_1$ than $k_2$ and prevent gel network formation. Thus, a balance of attractive and repulsive forces between polypeptide chains is critical for gel network formation with solvent conditions playing an important role (Ferry 1948).

**Recent gelation models**

It is fascinating that the gelation model proposed by Ferry in 1948 is still partially valid today. However, much more about globular protein gelation has been discovered since 1948 and his model no longer fits with current denaturation models. Ferry’s model only shows one unfolded state for denatured proteins, however, it presents a good starting point to separate the kinetics of denaturation ($k_1$) from kinetics of aggregation ($k_2$). Moreover, it can be used to explain gelation of gelatin and some chemically denatured globular proteins (Gilsenan and Ross-Murphy 2000).
Barbu and Joly (1953) and Tombs (1970) proposed a new way of looking at the mechanism of gelation. It has been proven that it is not necessary for globular proteins to unfold into “long chains” to form gel networks. Gel networks can be formed by both partially unfolded protein molecules as well as fully denatured proteins (Barbu and Joly 1953; Tombs 1970). However, a certain degree of change in protein structure (unfolding) is still required (Clark and Lee-Tuffnell 1986).

Recently, a model of protein folding and aggregation of polypeptide synthesis was proposed by Dobson (2003) and Chiti and Dobson (2006) (Fig 9). This model is based on a biological perspective focusing on miss-folding of proteins which results in amyloid fibrils. However, it can be used to understand and predict the denaturation and aggregation of native proteins since investigating heat-induced gelation of protein is similar to investigating the reverse pathway of protein folding (Foegeding 2006).

A simplified protein denaturation and aggregation model proposed by Foegeding (2006) (Fig 10) shows that native proteins go through an “intermediate” state before reaching the “unfolded” state. Both the intermediate and unfolded states have reactive sites which can form disordered aggregates. Three different types of aggregates can be formed: $\text{Aggregation}_{\text{intermediate}}$, $\text{Aggregation}_{\text{unfolded}}$, $\text{Aggregation}_{\text{amyloid}}$. It is important to note that the Foegeding (2006) model illustrates that “amyloid fibril” is a result of aggregation of proteins in the completely “unfolded” state based on the result of Ramen spectra (Ikeda and Li-Chan 2004). Whereas Dobson’s model predicts that “amyloid fibril” is a result of aggregation of proteins in an “intermediate” state.
Thus, a simple definition of heat-induced protein gelation is an aggregation of
denatured protein molecules which results in the formation of a continuous three dimensional
network. Note that only two molecules are required to form an aggregate, but a much higher
concentration is needed to form a gel network. Conformational change in the denaturation
step is a change in native structure with no changes in primary structure (Hermansson 1978),
therefore in principle, the change is reversible. However, unfolding exposes reactive groups
which results in the potential of an irreversible aggregation via covalent and non-covalent bonds.

![Simplified model of protein denaturation and aggregation model proposed by Foegeding (2006) as adapted from Dobson (2003).](image)

Effect of electrostatic stabilization on protein aggregation and microstructure of globular protein gels

Considering that the biggest factor preventing aggregation and gelation is electrostatic repulsion (Ferry 1948; Foegeding and others 1995; McGuffey and Foegeding 2001), gel structure and appearance depend on solution conditions (i.e., pH and ionic strength). Conditions that favor electrostatic interactions (i.e., pH close to pI and high ionic strength) are associated with a lower degree of protein unfolding. As pH moves away from pI (at low ionic strength), the proteins unfold more extensively due to an increase in electrostatic
repulsion, thereby preventing aggregation. Aggregation rate is increased with addition of salt. In addition, the reactivity of the sulfhydryl group also increases at high pH value.

It is known that the gel structure of WP gels is determined by solvent conditions. Based on electron microscopy images and rheological properties, WP gels have been characterized as “fine-stranded”, “particulate”, or “mixed” gels (Clark and others 1981; Errington and Foegeding 1998; Stading and others 1993). “Fine-stranded” gels are formed under the conditions favoring electrostatic repulsion (Fig 11). Gel networks formed under these conditions have strand diameters equal to the length of about one molecule and could appear to be curved or rod-shaped. These gels have structures that are initially apparent on the nano-scale and are translucent in appearance due to low light scattering of the thin protein strands (Barbut 1995). On the other hand, “particulate” gels are formed under the conditions where electrostatic interactions are low (Fig 11) (Langton and Hermansson 1992; van der Linden and Foegeding 2009). “Particulate” gels are composed of large aggregates resulting in opaque gels (Langton and Hermansson 1992) which are related to protein phase separation (Clark and others 1981). The phase separation stops when dense protein domains reach a few micrometers in size and can be observed at the microscopic level. More information regarding micro-phase separated gels can be found in the latter section.
Protein-polysaccharide interactions

Interactions of proteins and polysaccharides (biopolymers) create a wide range of gel structures which determine the texture of soft-solid gel based food products. Mixing biopolymers (i.e., proteins and polysaccharides) in an aqueous solution can result in various outcomes depending on the molecular properties of the biopolymers (i.e., charge density, MW, solubility, and chain stiffness) and solvent qualities (i.e., pH and ionic type/强度). Below the critical concentration for gelation (C₀), co-soluble, segregative, or associative phase separations are formed. Co-soluble results in an equal distribution of the biopolymers in the solvent. Mixtures of biopolymers often result in attractive or repulsive interactions resulting in associative or segregative phase separation, respectively. As a result, macroscopic phase separated two layer systems are observed (Fig 12). Biopolymer incompatibility is increased under conditions that promote protein self-association (i.e., pH close to pI and high ionic strength) and when the polymers have low affinity towards the solvents (Piculle and Lindmann 1992; Turgeon and others 2003).
Figure 12. Three outcomes of protein and polysaccharide mixtures in solution below critical concentration for gelation (de Kruif and Tuinier 2001).

Associative and segregative phase separations can be viewed based on entropy and enthalpy considerations (de Kruif and Tuineir 2001). Entropy driven phenomena are related to a difference in the biopolymer conformations. Enthalpy driven systems, on the other hand, are related to biopolymer chemical compositions dictating their solvent affinities.

Associative phase separation favors intermolecular interactions between two different biopolymers forming complexes and resulting in a phase that contains both types of biopolymers. Associative phase separation is considered to be entropy-enthalpy driven; complex formation is an entropic driven system in combination with electrostatic interactions which are enthalpic driven (de Kruif and Tuineir 2001). Segregative phase separation is caused by thermodynamic incompatibility between the biopolymers resulting in a phase separated system. Incompatibility is a fundamental property of many proteins and polysaccharides mixtures due to the differences in their molecular properties and conformation (Grinberg and Tolstoguzov 1997; Beaulieu and others 2001; Turgeon and Beaulieu 2001, Tolstoguzov 2003). Briefly, when proteins reach a critical size, they experience an induced depletion interaction due to an unbalanced osmotic pressure arising
from the exclusion of polysaccharide chains at the surface of the protein aggregates. Depletion interactions increase a mutual attraction between the protein aggregates and accelerate their growth rate (Croguennoc and others 2001). Above C_o, protein/polysaccharide mixed gels are formed which appear homogeneous at the macroscopic length scale since phase separation stops at a microscopic level. The mixed gels usually result in three different phase behaviors: interpenetrating, associative, and segregative phase separation (Tolstoguzov 1991; Piculelle and Lindman 1992; Turgeon and others 2003). Interpenetrating networks are observed when the two component gels form continuous independent networks (Morris 1986). Associative phase separation results in biopolymer complex networks. Segregative phase separation results in phase separated gel networks where one phase is rich in polysaccharides and the other is rich in proteins with partition of solvent between phases.

Phase separation is a kinetic process (Turgeon and Beaulieu 2001; Turgeon and others 2003), therefore the kinetics of gelation versus phase separation are important parameters that dictate the final microstructure of mixed gels. Phase separation of protein/polysaccharide mixtures only happen when the mixture is still a liquid and no further change in microstructure is observed once the system becomes a gel (de Jong and others 2009). Therefore, other factors that affect gel formation and phase separation, other than molecular properties of the biopolymers and solvent conditions, should be considered as determining factors of the microstructures. This includes processing parameters such as mixing procedures (i.e., shear rate) and heating conditions (i.e., heating rate) (Edelman and
van der Linden 2001; Turgeon and Beaulieu 2001; Turgeon and others 2003; de Jong and van de Velde 2007).

Formation and properties of whey protein/polysaccharide mixed gels have been investigated (van den Berg and others 2007, de Jong and van de velde 2007; de Jong and others 2009; Cakir and Foegeding 2011). The effect of their interactions on the microstructure and rheological properties is reviewed in detailed in the next section.

**Gel structure: Micro-phase separation**

Food structure can be defined as the way molecules are organized in food products. Different levels of structure (macro, micro, sub micron, and nano) are important in determining food texture. Food texture is one of the most important sensory attributes determining consumer perceptions and acceptability of foods (Bourne 1978). Therefore, a good understanding of how food structure is created, stabilized and broken down is important when relating food texture to sensory perception of texture. Confocal Laser Scanning Microscopy (CLSM) and Scanning Electron Microscopy (SEM) are commonly used to study the structure of soft-solid gels at micro- and sub micron length scale, respectively (van den Berg 2007, 2009; de Jong and van de Velde 2007; Çakir and Foegeding 2011).

Unlike macro-phase separation where inhomogeneous structure is an outcome, often associated with a defect in the products, micro-phase separation appears homogenous at the macro-length scale. This is because gel formation stops phase separation of mixed gels at a micro level before it reaches equilibrium in a macroscopic level. However, some structures can be observed at the microscopic level.
This section of literature review is focused on structures of protein gels and protein/polysaccharide mixed gels at the microscopic level. The terms “micro-phase separation” and “single phase” refer to heterogeneous and homogeneous gel structures, respectively, observed at length scale of 1µm and greater (van den Berg and others 2007; Ako and others 2009). In addition, a brief review on how these structures determine gelation properties and sensory perception of the gels will also be covered.

**Micro-phase separation: Protein**

Ako and others (2009) provided a new perspective on viewing microstructure of globular protein gels. Light and x-ray scattering in combination with CLSM images were used to determine the effect of solution conditions and protein concentration on the phase stability of β-lactoglobulin allowing for construction of a state diagram model (Fig 13).

![State diagram relative to protein (x-axis) and salt (y-axis) concentration (Ako and others 2009).](image)

Figure 13. State diagram relative to protein (x-axis) and salt (y-axis) concentration (Ako and others 2009).
The key concept established was that the system can be in single phase or two phase regions depending on solution conditions and protein concentration. Under high electrostatic stabilization conditions, the system is considered to be in a single phase; protein aggregates remain soluble and dispersed forming a “Sol” when heated at concentration below $C_0$. Above $C_0$, denatured proteins form “homogenous” gels which appear as a single phase at the micro length scale (Fig 14 A and F). On the other hand, when the electrostatic stabilization is low, attractive forces lead to aggregation of proteins, moving the system into the two phase region. In this region, proteins undergo slow aggregation even without denaturation. Heating solutions produced protein “precipitate” below $C_0$ and formed “micro-phase separated” gels above $C_0$. The structure appears to be heterogeneous and thus called “micro-phase separated” (Fig 14B to E). Micro-phase separated structure is a result of a competition between Stoke’s based setting of large aggregates versus aggregates forming into a gel network. Factors affecting the kinetics of aggregation and settling (i.e., heating rate, protein charge density) will determine the degree of homogeneity of the gel structure.

Moreover, Ako and others (2009) showed that there is a pH range where the system moves from single phase to two phase region as the system move towards the pI of the
protein. It is important to note that this region is not just at the pI of β-lg (pH 5.2) but it occurs over a range of pH 4.1 to 5.6 (Fig 14 and 15). Their study clearly showed how pH relative to protein pI and solvent ionic strength determine gel types (Fig 14 and 16). However, the micro-phase separation model proposed by Ako and others (2009) is based on a single protein, β-lactoglobulin (β-lg).

Figure 15. The effect of pH on A. Apparent molar mass, B. Correlation length, C. Turbidity of 10% (w/v) of β-lg gels. Data from: ○ Light scarring, ● CLSM (Ako and others 2009).

Kitabatake and others (1995) investigated the effect of ionic strength and pH on turbidity of heat-induced 7% (w/v) ovalbumin and egg white protein (EWP) gels. Absorbance at 590 nm was used as an indicator of turbidity. Turbidity values of ovalbumin and EWP gels relative to pH and NaCl concentrations are presented in contour graphs (Fig 16B and C). At pH close to pI of ovalbumin (pH 4.5), the gels are more opaque and the ranges broaden as the salt concentration is increased. The shape of the phase diagram of β-lg reported by Ako and others (2009) shows trends similar to the turbidity diagram of ovalbumin. Although the transition between transparent and opaque gels are not the same as the transition between single and micro-phase separated gels, the results are comparable. It
was shown that the turbidity increases before the system moved into the micro-phase separated region (Ako and others 2009).

![Figure 16](image)

Figure 16. A. State diagram shows how solvent conditions (x-axis) affect the microstructure of β-lg gels at different salt concentration (y-axis) (Ako and others 2009). B. Turbidity contour graphs show how solvent conditions (x-axis) affect ovalbumin; C. Egg white protein gels (Kitabatake and others 1995). Note: figure B and C are rotated to compare with the state diagram of β-lg gels and the line numbers indicate the optical density value.

Moreover, ovalbumin shows less requirement of salt to induce aggregation than β-lg. This trend was also observed using light scattering measurement (Kitabatake and Kinekawa 1995; Ako and others 2009). The results of β-lg and ovalbumin phase diagram suggest the effect of salt concentration and pH on the phase diagrams of different globular proteins are similar but the proteins and ionic strength/type will determine the exact condition for phase separation. However, more work is needed to explain the cause of micro-phase separation of protein ingredients (which are comprised of more than just one protein). The shape of the turbidity diagrams of heat-induced ovalbumin (Fig 16B) and EWP (Fig 16C) also show similar trends but EWP gels require much less salt to induce aggregation than ovalbumin (Kitabatake and Kinekawa 1995).
**Micro-phase separation: protein-polysaccharide**

Above critical concentration for gelation, biopolymer mixtures can result in associative or segregative phase separated networks (Doublier and others 2000; de Kruif and Tuinier 2001). Phase separation is the most common outcome of mixed gels (Morris 1986). More details regarding protein/polysaccharide interactions can be found in section 4. The microstructure of mixed gels has been characterized into “homogenous”, micro-phase separated (“protein continuous”, “bicontinuous”, and “coarse stranded”) and “polysaccharide continuous” (van den Berg and others 2007). “Protein continuous” structure is made up of a continuous protein network with dispersed discontinuous droplets (approximately 10 µm) of polysaccharide and water, known as “serum”. “Coarse stranded” and “bicontinuous” structures are similar but the main difference is the thickness of protein strands, 1 to 3 µm versus 3 to 15 µm, respectively (van Vliet and others 2009). Examples of the different microstructures of WPI/polysaccharide mixed gels are shown in Figure 17. These microstructures suggest that nomenclature of mixed gels need to be more well defined since the bicontinuous and polysaccharide continuous structures from these two studies appear to be different.
The degree of micro-phase separation of WPI/polysaccharide mixed gels has been shown to depend on the polysaccharide’s molecular character, concentration, solution conditions and gelation conditions (de Jong and van de Velde 2007; de Jong and others 2009; Çakır and Foegeding 2011). For example, under the conditions where the structures of WPI gels appear in the single phase (pH 7.0 and 50 mM NaCl), the structure of the mixed gels changed from “homogenous” to “protein continuous” to “bicontinuous” and finally “polysaccharide continuous” as κ-carrageenan concentration increased from 0 to 0.6% (w/w) (Fig 18; top row). While under the two phase region (pH 5.5 and 100 mM NaCl or pH 7.0), the structure changed from “particulate” to “coarse stranded” to “polysaccharide continuous” as κ-carrageenan concentration increased (Fig 18; bottom row) (Çakır and Foegeding 2011; Çakır 2011).
Globular proteins form homogenous gels above $C_o$ under high electrostatic stabilization conditions ($\text{pH} \gg \text{pIs}$) (Fig 14). When polysaccharides are added under these conditions, a clear demarcation is observed between protein aggregates and the serum phase (Fig 19; pH 7.0 and 8.0). As pH moves toward the pI of the proteins, clear demarcation was no longer observed with polysaccharide addition (Fig 19 pH 5.0 to 6.5). Figure 19 shows how protein charge density ($\text{pH}$) alters the microstructure of the mixed gels. As the pH of the system approaches the pI of the protein, protein-protein interactions are favored (Ako and others 2009) which increases the incompatibility between the biopolymers (de Kruif and Tuinier 2001; Piculell and Lindmann 1992). Addition of polysaccharides further increases protein aggregation and induces formation of a coarse microstructure.
Another example of how biopolymer molecular characteristics, in this case charge density of the polysaccharides, affect the microstructure of mixed gels is shown in figure 20 (de Jong and van de Velde 2007). Addition of lower charge density polysaccharides resulted in a higher degree of micro-phase separation than when higher charge density polysaccharides were added (Fig 18).

**Gel structure explains gelation properties (fracture and water holding properties) and sensory perception**

Gel microstructures have been shown to correlate well with water holding, fracture properties, and breakdown pattern (crack propagation speed within the material) of the gels which determine the overall sensory texture perception (Gwartney and others 2004; Çakır and others 2011; van den Berg and others 2008). Thus, a good understanding of food texture begins with understanding the microstructure.
Figure 20. Effect of polysaccharide charge density on the microstructure of acid-induced WPI/polysaccharide mixed gels (Modified from de Jong and van de Velde 2007).

Water holding properties are related to gel porosity and are one of the most important factors for determining how breakdown gels and their texture is perceived during consumption (van den Berg and others 2007). Breakdown pattern has been shown to relate to crumbliness and spreadibility properties. “Crumbliness” is the term used to describe how a sample falls apart in the mouth. It is one of the characteristic sensations of products such as cheese, processed meat (e.g. hot dog) or confections which determine consumer acceptance and is commonly used as a parameter for quality control (van den Berg and others 2008). Breakdown pattern is an indirect measurement of the elastic properties of viscoelastic materials since energy stored and dissipated in the material determines fracture speed (van den Berg and others 2008). Gels with high elastic components enable them to use the elastically stored energy to fracture via a free-running crack during large deformation resulting in fast fracture and steep breakdown patterns. These gels are known to be highly
“crumbly”. On the other hand, gels with a high viscous component show yielding behavior and as a result have slow fracture propagation (van den Berg and others 2008; Çakır and others 2011). These gels are known to have high “spreadibility” properties and often release large amounts of water upon deformation (van den Berg and others 2008).

Protein gels

“Homogeneous” protein gels break down into large inhomogenous particles with small amounts of water released during deformation. They are characterized as springy with smooth and slippery surfaces. Moving from “homogeneous” to “particulate” microstructure results in decreased water holding and changes in fracture properties (Çakır and Foegeding 2011). “Particulate” protein gels breakdown into small homogenous particles that release a high amount of water during mastication (van den Berg and others 2007; Çakır and others 2011). Breakdown patterns of “particulate” WPI gels were more gradual than “homogenous” WPI gels (Çakır and others 2011). Pores and particle size determine gel breakdown properties and grittiness, respectively (Langton and others 1997). Increase in pore size results in lower strength due to a reduction in fracture surfaces (Walstra 2003), whereas increasing the particle size increases the perception of grittiness.

“Fine-stranded” WP gels observed under nano length scale formed at low ($\leq 3.5$) and high ($\geq 6.5$) pH have different textural properties; gels formed at low pH are weak and brittle, whereas gels formed at high pH are strong and elastic (Tang and others 1994; Errington and Foegeding 1998). At high pH, reactivity of sulphydryl groups is increased however, disulfide interactions were shown to have no impact on the fracture properties of the gel. Addition of
disulfide blockers only alter gel properties in the non-linear region which illustrates that fracture properties are determined by electrostatic interactions (McGuffey and Foegeding 2001).

**Protein-polysaccharide mixed gels**

Protein and polysaccharide interactions determine the microstructure of soft-solid gel based food products. Different microstructures control the gelation properties and textural sensory of mixed gels. Gel strength has been shown to mainly relate to the local protein concentration of the continuous phase, while water release during deformation has been shown to mainly relate to porosity based on Darcy’s equation (van den Berg and others 2007; Çakır and Foegeding 2011). Connectivity among protein aggregates is enhanced as the local protein concentration increases which leads to both an increase in gel stiffness and gel strength where higher force is needed for deformation and fracture (Çakır and others 2011). A dense and firm gel made up of 12% β-lactoglobulin has higher fracture stress when compare to 6% β-lactoglobulin gels (Öhgren and others 2004).

In addition, “homogeneous” and “protein continuous” mixed gels were shown to fracture in one distinct plane and to have low water release which is associated with rapid breakdown of the sample. These mixed gels were shown to be firm with high crumbliness scores. Conversely, “coarse stranded” and “bicontinuous” mixed gels showed yielding behavior because they fractured at multiple points in the micro-length scale during compression which produced a large number of pieces and were perceived as low crumbly and high spreadable gels (van den Berg and others 2008).
**Heating rate effects**

**Effect of heating rate on gelation properties of globular protein gels**

Rapid heating methods are advantageous for reasons of increased throughput and reduced energy waste. The effect of heating rate on physical properties of different proteins has been studied; β-lactoglobulin and whey proteins (Stading and Hermansson 1992, 1993; Li and others 2006), bovine serum albumin (Foegeding and others 1986), meat proteins (Barbut and others 1990; Camou and others 1989; Foegeding and others 1986; Riemann and others 2004), and legume protein (Renkema 2004; Sun and Arntfield 2011). However, the results showed contradicting trends. Some works showed that faster heating produced more rigid and stronger gels, while the others reported the opposite. It is difficult to compare among studies because they were conducted under different solution conditions (i.e., pH and ionic strength) and received different total thermal input (integration of time and temperature). The results of these studies regarding heating rate are summarized in the first manuscript as shown on page 69.

Based on the two-step gelation model proposed by Ferry (1948) (Fig 8), one explanation for lower gel strength and rigidity as a result of fast heating rate is insufficient time for proteins to denature prior to aggregation and gelation (Hermansson 1978). Contrary to this, Foegeding and others (1986) proposed the concept that aggregation is the limiting step during fast heating. This is based on denaturation being a very rapid process. Thus, weaker gels produced by fast heating rates were due to not allowing sufficient time for proteins to aggregate.
Torley and Young (1995) used a combination of dynamic and isothermal heating, pausing and holding at one of several temperatures (ranging from 25 to 55 °C) for 180 min before achieving end-point temperature, to determine rheological changes of salted beef homogenates during heating. All heating regimes produced gels of essentially identical rigidity and elasticity, regardless of the thermal path. Riemann and others (2004) investigated the effect of heating rate (0.5, 20, and 98 °C/min and 20 min hold) on gelation of Alaskan pollock surimi and turkey breast. Gels prepared by slower heating rates exhibited the highest gel strength when compared at a common end-point temperature. However, if given enough time for aggregation after reaching end-point temperature, gels prepared by rapid heating were as strong as those prepared at a slow heating rate (Riemann and others 2004).

These studies suggest that as long as the denaturation temperature ($T_d$) is reached and sufficient time is allowed for aggregation above $T_d$, gel formation and properties will be similar. Aggregation time and total thermal input (integration of time and temperature) are the key thermal processing parameters (Foegeding and others 1986; Riemann and others 2004). A simple model proposed for formation of particulate gels by Verhuel and Roefs (1998) supports this finding. The amount of protein incorporated into the gel network at the gel point determined the gel network microstructure and water holding properties. After gel point was reached, rearrangements within the protein network occurs and/or more proteins are added to the network which causes no change in the water holding properties or structure but do increase gel rigidity and strength (Verheul and Roefs 1998) (Fig 21).
Recently, Sun and Arntfield (2011) investigated the effect of heating (0.5 – 4 °C/min; a 0 min hold, 300 mM salt) on gelation properties of salt-extracted pea protein isolate and reported that there is an optimum heating rate (2 °C/min) which results in maximum firmness. Heating rapidly resulted in lower rigidity which was explained by insufficient time allowed for proteins to aggregate. However, they were not able to explain the reason for the low gel rigidity obtained when too slow of a heating rate was used.

Based on micro-phase separation and aggregation limited models proposed by Ako and others (2009) and Foegeding and others (1986), respectively, when the system is under micro-phase separation region there is an optimum heating rate that results in gels with maximum rigidity. Under this region, slow heating rates do not always produce gels with the highest rigidity, due to protein precipitation. Without subsequent holding, there is an optimal heating rate that is slow enough for proteins to maximize network rearrangement while allowing networks to form prior to precipitation due to Stokes’ consideration. If this
explanation holds true, slow heating rates should not cause protein precipitation when the system is under a single phase.

**Effect of heating rate on the gelation properties of protein/polysaccharide mixed gels**

Mixtures of biopolymers result in phase separation (see more detail on p. 47). Phase separation of protein/polysaccharide mixtures only happens when the mixture is still a liquid and no further change in microstructure is observed once the system becomes a gel (de Jong and others 2009). Gel microstructures are a result of a competition between phase separation versus gelation and the factors that affect the kinetics of gel formation or phase separation should be considered as factors for determining the microstructures of the mixed gels (Ould Eleya and Turgeon 2000; Turgeon and Beaulieu 2001; Turgeon and others 2003; de Jong and others 2009) (Fig 22).

![Diagram of protein/polysaccharide mixed gel](image)

Figure 22. Schematic of heat-induced protein/polysaccharide mixed gel.

The effects of heating rate on microstructures of WPI/LM pectin mixed gels were observed at three different pH values (Turgeon and Beaulieu 2004) (Fig 23). Clearly,
biopolymer molecular properties (i.e., protein charge density) and heating rate are important factors determining the microstructure of the mixed gels. Altering the heating rate will affect mixed gel formation since it slows down or speeds up the protein aggregation and gelation (Li and others 2006).

Figure 23. Effect of heating rate on microstructure of WPI/LM pectin mixed gels at various pHs. Fast heating rate (6 °C/min) slow heating rate (1.3 °C/min) (Turgeon and Beaulieu 2004).

In cold set protein gelation, acidification rate controls the rate of gelation similarly to how heating rate controls the rate of gelation of heat-induced protein gels (Fig 24). The rate of acidification was also shown to change the microstructures of the mixed gels (de Jong and others 2009). A coarser microstructure is formed when slower acidification rates are used allowing more time for proteins to aggregate and phase separation to occur (de Jong and others 2009).
Figure 24. Microstructure of WPI/LBG mixed gels at different rates of acidification. Left: 0.25 pH/hr; Right: 0.06 pH/hr. Image size: 160 x 160 µm (de Jong and others 2009).
Impetus for this research

A review of literature indicates that extensive work in the area of globular protein gelation has been done. Many gelation models were developed over the past decades to understand the physico-chemical properties of protein gels. Gel structure was shown to be a key concept in understanding these properties and ultimately the texture of food products. Recently, a micro-phase separation model proposed by Ako and others (2009) provided a new perspective on the structure of protein gels based on solution conditions favoring either single phase (stranded) or micro-phase separated aggregates (particulate). Different structures can be create by altering processing condition (i.e., heating rate), solvent condition (i.e., pH), and composition (i.e., polymer mixtures). Egg white protein and whey protein isolate are different in their compositions, molecular properties, and respond different to changes in these parameters. The objective of this research was to understand the mechanisms responsible for structural formation and gelation properties of globular protein gels based on micro-phase separation consideration.
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CHAPTER 2
MANUSCRIPT 1

How Micro-Phase Separation Alters the Heating Rate Effects on Globular Protein Gelation

This manuscript has been published in:


Authors: Phanin N Leksrisompong and E. Allen Foegeding
How Micro-Phase Separation Alters the Heating Rate Effects on Globular Protein Gelation

Phanin N. Leksrisompon and Edward Allen Foegeding

Abstract: This study was conducted to determine how the combination of heating rate and pH can be used to alter viscoelastic properties and microstructure of egg white protein and whey protein isolate gels. Protein solutions (1% to 7% w/v protein, pH 3.0 to 8.5) were heated using a range of heating rates (0.2 to 60 °C/min) to achieve a final temperature of 80 °C. The gelation process and viscoelastic properties of formed gels were evaluated using small strain rheology. Single phase or micro-phase separated solution conditions were determined by confocal laser scanning microscopy. In the single phase region, gels prepared by the faster heating rates had the lowest rigidity at 80 °C; however, a common G' was achieved after holding for 4 h at 80 °C. On the other hand, under micro-phase separation conditions, faster heating rates allowed phase separated particles to be frozen in the network prior to precipitation. Thus, gels produced by slower heating rates had lower rigidities than gels produced by faster heating rates. The effect of heating rate appears to depend on if the solution is under single phase or micro-phase separated conditions.

Keywords: egg white, gelation, heating rate, micro-phase separation, whey protein isolate

Practical Application: The effect of heating rate and/or time on protein gel firmness can be explained based on protein charge. When proteins have a high net negative charge and form soluble aggregates, there is no heating rate effect and gels with equal firmness will be formed if given enough time. In contrast, when electrostatic repulsion is low, there is a competition between protein precipitation and gel formation; thus, a faster heating rate produces a firmer gel.

Introduction

One of the main functionalities of proteins is their ability to form gels upon heating. Heat-induced globular protein gels form 2 general types of networks: those formed from linear or rod-like aggregates (stranded) and those made with particulate aggregates (particulate) (van der Linden and Foegeding 2009). The 2 types of heat-induced protein gels have different rheological, microstructural, and water-holding properties. These properties are affected by protein origin, protein concentration, solution conditions (pH and ionic strength), and gelling procedures such as heating temperature, time, and rate (Clark and Lee-Tuffnell 1986; Arrnfield and others 1989; Mulvihill and others 1990; Langton and Hermansson 1992; Bowland and Foegeding 1995). Of these factors, pH is also important because it is used to classify foods and determine the type of thermal processing. A pH lower than 4.6 is considered to be high acid foods and vice versa (Fellows 1988).

Protein aggregation/gelation is very pH dependent. It influences intermolecular interactions through changing the electrostatic properties as well as affecting chemical reactivity (i.e., thiol groups) of the proteins and, in turn, impacts the type of network formed and their physical properties. Physical properties of gels can also be altered by heating rates through the relationship between protein denaturation and aggregation (Ferry 1948; Arnfield and others 1989). Previous investigations have shown that heating rate affects the physical properties as well as microstructure of the protein gel; however, there is disagreement in how heating rate alters physical properties. Some researchers reported that fast heating produced more rigid gels (Stading and others 1992), while other researchers reported the opposite results (Foegeding and others 1986; Arnfield and Murray 1992; Stading and others 1993; Langton and Hermansson 1996; Li and others 2006). It is difficult to compare among studies because they were conducted at different solution conditions (e.g., pH and ionic strength). In addition, some studies only compared gels that were cooked to a common endpoint temperature without considering the effect of integration of time and temperature. Table 1 is a summary of the results of all the studies regarding heating rate that are discussed in this paper.

Ferry (1948) proposed a 2-step protein gelation model with the 1st step being denaturation and exposure of sites for intermolecular interactions and the 2nd being aggregation to form a gel (Figure 1). It should be noted that this model implies just one structure for a denatured protein that does not fit with current denaturation models predicting more than one unfolded state. Nonetheless, it presents a starting point to separate the kinetics of denaturation from aggregation. Based on Ferry’s 2-step model, Foegeding and others (1986) proposed that fast heating rates to a common endpoint allowed less time for proteins to spend at temperatures above the denaturation temperature. Less rigid gels produced by fast heating were due to insufficient time for aggregation rather than insufficient time for denaturation. This is based on denaturation being a very rapid process. A kinetic study of folding and unfolding of α-helix and β-sheets of 2 naturally occurring variations of B domain of protein G (Gπ) showed that the

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Table 1: A summary of the results of ours and other researcher studies on the effect of heating rate on rheological and microstructure of protein gels.

<table>
<thead>
<tr>
<th>Authors and others (1996)</th>
<th>Protein type</th>
<th>Protein concentration</th>
<th>Ionic type and strength</th>
<th>pH</th>
<th>End point T (°C)</th>
<th>Holding time (min)</th>
<th>Heating rate range (°C/min)</th>
<th>Measurement</th>
<th>Results</th>
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<tbody>
<tr>
<td>Frodlings and others</td>
<td>Myosin</td>
<td>3%, 6% w/v</td>
<td>300 mM NaCl and 0.1M NaN3</td>
<td>6.0</td>
<td>70</td>
<td>0</td>
<td>0.2-0.6, and constant water temperature for 20 min</td>
<td>Back extension</td>
<td>Slow heating rate produced stronger gels than a constant heating rate</td>
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<td>Fennema</td>
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<td>Abumir</td>
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<tr>
<td>Arnfield and Marz (1992)</td>
<td>Ovalbumin</td>
<td>10% w/v</td>
<td>150 mM NaCl</td>
<td>5.0</td>
<td>95</td>
<td>2</td>
<td>0.5 to 3 °C/min</td>
<td>Viscelastic properties</td>
<td>No significant effect of heating rate</td>
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<td>Veldin</td>
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<td>Viscelastic properties</td>
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<td>Stading and others (1995)</td>
<td>β-lactoglobulin</td>
<td>12% to 14% w/v</td>
<td>-</td>
<td>7.5</td>
<td>90</td>
<td>0</td>
<td>0.017 to 12 °C/min</td>
<td>Viscelastic properties</td>
<td>Faster heating rate results in lower G'</td>
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<td>Stading and others (1995)</td>
<td>β-lactoglobulin</td>
<td>10% w/v</td>
<td>-</td>
<td>5.3</td>
<td>90</td>
<td>0</td>
<td>0.1 to 12 °C/min</td>
<td>Viscelastic properties</td>
<td>Faster heating rate results in lower G’</td>
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<tr>
<td>Langton and Hermansson (1996)</td>
<td>Whey protein concentrate</td>
<td>13.5% w/v</td>
<td>0 to 0.1 mol/dl</td>
<td>4.6 to 5.4</td>
<td>90</td>
<td>0</td>
<td>1.3</td>
<td>Microstructure (SEM-TEM)</td>
<td>Gel formation at the slow heating rate consisted of a homogeneous network with poor sizes of 20 to 30 pm. At a slow heating rate, the network bed formed porc 90 to 150 and was inhomogeneous and broader distribution of particle size</td>
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<td>Barns and others (2004)</td>
<td>Turkey and dried Pollock trimm</td>
<td>2% NaCl</td>
<td>-</td>
<td>7.0</td>
<td>90</td>
<td>0</td>
<td>0.1 to 20 °C/min</td>
<td>Fracture properties</td>
<td>Without subsequent holding prior to cooling, gel prepared by slow heat exhibit monoger gel strength for both turkey and salmon. However, gel prepared by rapid heating at a 20 min holding period were stronger as gel prepared by slow heating for both species</td>
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<tr>
<td>Li and others (2006)</td>
<td>Whey protein isolate</td>
<td>15% w/v</td>
<td>0.1M Phosphate buffer</td>
<td>7.0</td>
<td>90</td>
<td>0</td>
<td>0.1 to 20 °C/min</td>
<td>Viscelastic properties</td>
<td>Faster heating rate results in lower G’</td>
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<tr>
<td>Bradley and others (2006)</td>
<td>β-Lactoglobulin</td>
<td>3% w/v</td>
<td>-</td>
<td>5.3</td>
<td>84</td>
<td>1</td>
<td>1 to 100 °C/min</td>
<td>Microstructure (environmental SEM)</td>
<td>Faster heating rate results in lower polydispersity and uniform particle size</td>
</tr>
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</table>

74
unfolding of G_H and G_R occur rapidly; having half-lives of 6.18 and 0.15 ms, respectively, at 35 °C (Alexander and others 1992b). The G_H domain is small compared to other proteins but the thermodynamics of the folding reaction are similar to larger globular proteins (Alexander and others 1992a). This supports the concept that aggregation is the limiting reaction. Verheul and Roefs (1998) proposed a model for formation of particulate gels that establishes the gel network microstructure type at the gel point, followed by a slow development of rigidity over time. Post gel point, rearrangements within protein network occur and/or more proteins are added to the network that causes an increase in gel rigidity but no change in structure type.

Riemann and others (2004) investigated the effect of heating rate on gelation of Alaskan Pollock surimi and turkey breast. Gels were formed by heating at 0.5 °C/min, 20 °C/min, or 98 °C/min to 70 °C. Without subsequent holding at 70 °C prior to cooling, gels prepared by slower heating rates exhibit higher gel strength for both turkey and surimi. However, gels prepared by rapid heating and a 20 min holding period were as strong as gels prepared by slower heating for both species. This showed that integration of time and temperature was the key thermal processing parameter. The findings of Riemann and others (2004) suggest that the differences in heating rate were simply due to not allowing for sufficient time to have the same final gel structure formed.

Recently, Ako and others (2009) provided a new perspective on the structure of protein gels based on solution conditions favoring either single phase or micro-phase separated aggregates, allowing for construction of a state diagram for gelation of β-lactoglobulin. This clearly showed how pH relative to protein isoelectric point (pI) and solvent ionic strength determine gel types (e.g., strained vs. particulate). The key concept established was that the electrostatic stabilization of proteins increases, the system moves away from a micro-phase separation region. In single phase conditions, at protein concentrations above the critical concentration for gelation (C_c), denatured protein will form a homogenous gel. On the other hand, when the system is in the micro-phase separated region, particulate aggregate gels are formed and gel structure is the result of a competition between large aggregates setting due to Stokes’-based considerations versus frozen in the network prior to precipitation. This suggests that heating rate (or time) effects will be seen in low-viscosity globular protein solutions when solvent conditions put the system in the micro-phase separated region.

The objective of this study was to determine how the combination of heating rate and pH alter viscoelastic properties and microstructure of egg white protein (EWP) and whey protein isolate (WPI) gels. Results were interpreted based on the kinetic model of Ferry (1948) and the micro-phase separation model of Ako and others (2009).

Materials and Methods
Spray dried EWP (82.5% protein, dry basis) was obtained from Michael Foods, Inc. (Minnetonka, Minn., U.S.A.) and stored at 4 °C for less than 12-month period. WPI (94.21% protein, dry basis) was supplied by Davisco Foods Intl., Inc. (Le Sueur, Minn., U.S.A.). WPI was stored at room temperature (22 ± 2 °C) until used. Sodium hydroxide (ACS pellets) and sodium chloride were purchased from Fisher Scientific Inc. (Fair Lawn, N.J., U.S.A.). Hydrochloric acid was purchased from Mallinckrodt Baker Inc. (Paris, Ky., U.S.A.). Deionized water was obtained using a Dracor Water Systems (Durham, N.C., U.S.A.) purification system with a resistivity minimum of 18.2 MΩ-cm.

Protein solutions
Protein solutions were prepared by hydrating EWP or WPI in deionized water and stirring for 5 to 6 h at room temperature (22 ± 2 °C) to allow for full hydration. WPI solutions contained 100 mM NaCl to allow for maximum gel rigidity and strength (Ikeda and others 1999). The pH of EWP and WPI solutions were adjusted to 3.0, 4.5, 7.0, or 8.5 using 1 N sodium hydroxide or 1 N HCl prior to final adjustment to 1%, 5%, or 7% w/v protein and degassed for 1 h. The EWP solutions were centrifuged at 3216 x g for 30 min to remove insoluble components. Protein analysis (Kephichinomy acid, Pierce, Rockford, Ill., U.S.A.) showed no significant difference between samples before and after centrifugation indicating no significant loss of protein. The pH values were chosen to produce a range of gel microstructures. The strength of heat-induced EWP gels has been reported to be maximum around pH 8.5 (Hickson and others 1980; Holt and others 1984; Power and Nakai 1985).

Rheological measurement
The gelation process and viscoelastic properties of the formed gels were measured using a serrated cup and bob geometry attached to a stress-controlled rheometer (StressTech, Rheologica Instruments AB, Lund, Sweden). A protein concentration of 7% (w/v) was used to allow for sensitivity in detecting the gel point and differentiating among conditions. Stress sweeps were conducted to identify the linear viscoelastic region of each gel. Subsequent experiments were conducted on a stress of 1 Pa during gel formation followed by 50 Pa during holding and at a frequency of 0.1 Hz. Rheological properties (storage modulus, G' and loss modulus, G") were determined as protein solutions were heated from 20 to 80 °C at 20 °C/min, 2 °C/min, or 0.2 °C/min and held for 4 h. This was followed by a frequency sweep (0.001 to 100 Hz). All rheological tests were replicated 3 times. This range of heating rates was selected to be compatible with minimal possible heating rates on all instrumentation used in this study.

Gelation curves (G' development vs. time) were normalized based on gel time (Table 2) to evaluate possible master curves across heating time (Figure 2). Gel time was the time when G' reached a value of >10 Pa.

Visual observations of phase separation
Protein solutions (1% and 5% w/v) at pH 3.0, 4.5, 7.0, and 8.5 were filled into glass tubes and heated from 20 to 80 °C using a range of water bath temperatures (85 to 98 °C) for the 20 °C/min heating rate, or heated at 2 °C/min or 0.2 °C/min using a programmable water bath. Gels were cooled immediately after heating by immersion in an ice-water bath to decrease gel temperature to 4 ± 1 °C at a rate of approximately 10 °C/min. A qualitative assessment of the physical properties was determined by tilting the tube and observing change. "Viscous fluid" indicates flow; "Soft gels" was those that deformed but did not flow while "Gels" indicates no movement.
Turbidity measurement

A 2100AN Turbidimeter (HACH Co, Loveland, Co., U.S.A.) was used to measure turbidity of the heated solutions. The turbidity of the solutions was not measured when phase separation occurred.

Confocal laser scanning microscope

Gel microstructure was observed using a Zeiss LSM 710 confocal laser scanning microscope (CLSM) equipped with an inverted microscope (Zeiss Axio Observer Z1) in the fluorescence mode. The objective lens used was a 40x (LD C-Apochromat 40x/1.1 W Korr M27). Solutions were stained with an aqueous solution of Rhodamine B (for noncovalent labeling of the protein) prior to gelation (10 μL of a 0.2% (w/w) Rhodamine B + 1 mL of a 7% w/v protein solutions). The stained solution (50 μL) was pipetted into a glass bottom microwell dishes (35-mm petri dish, 14-mm Micro well, Nr 1.5 cover glass 0.16 to 0.19 mm (MaTech Corp., Mass., U.S.A.) and sealed with a top cover slip (modified method of Akio and others 2009). Samples were heated from 20 to 80 °C at a controlled heating rate of 2 °C/min or 0.2 °C/min. A faster heating rate was achieved by placing the sealed slide directly in the water bath at 80 °C for 3 min, which corresponds to an average heating rate of approximately 60 °C/min, following by holding at 80 °C for 2 min to allow for gelation. The excitation was at 514 nm and the emission of Rhodamine B was recorded between 546 and 700 nm.

Statistical analysis

Data were analyzed using analysis of variance using PROC GLM of SAS (version 9.1, SAS Inst. Inc., Cary, N.C., U.S.A.) and comparisons of the means using Tukey’s significant difference test (P < 0.05).

Results and Discussion

Rheological measurements—gel formation and viscoelastic properties

The effects of heating rate (0.2 to 20 °C/min) and holding time (4 h) on gelation of 7% (w/v) WPI or WPI solutions were investigated at pH 3.0, 4.5, 7.0, or 8.5 (Figure 2). Storage modulus (G’) and loss modulus (G”) values represent the respective elastic and viscous components in the network. For all the pH treatments, both G’ and G” developed similarly and the G’ values were significantly greater in magnitude than the G” values at 80 °C for all gels (Figure 2); therefore, only G’ values are reported. The

Table 2—Gelation times and temperatures of 7% (w/v) WPI and WPI gels.

<table>
<thead>
<tr>
<th>pH</th>
<th>Gelation temperature (°C)</th>
<th>Gelation time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.0</td>
<td>80</td>
<td>21*</td>
</tr>
<tr>
<td>4.5</td>
<td>80</td>
<td>32*</td>
</tr>
<tr>
<td>7.0</td>
<td>70</td>
<td>29*</td>
</tr>
<tr>
<td>8.5</td>
<td>70</td>
<td>25*</td>
</tr>
</tbody>
</table>

Whey protein isolate

2 °C/min: 80 °C, 21 * 4 °C/min: 80 °C, 32 * 0.2 °C/min: 70 °C, 29 *

Egg white protein

2 °C/min: 70 °C, 3 * 4 °C/min: 68 °C, 24 * 0.2 °C/min: 61 °C, 24 *

Different letters in each column, among heating rates within protein type, are significantly different (P < 0.05) from each other.

Figure 2—Normalized development of storage modulus (G’) over time for 3 different heating rates of 7% (w/v) WPI or WPI at various pHs: (A) WPI pH 3.0; (B) WPI pH 4.5; (C) WPI pH 7.0; (D) WPI pH 8.5; (E) WPI pH 3.0; (F) WPI pH 4.5; (G) WPI pH 7.0; and (H) WPI pH 8.5. Heating rates were 20 °C/min, 4 °C/min, 0.2 °C/min, 0. 
development of $G'$ was normalized by gel time (Table 2) in order to detect possible master curves across heating time (Figure 2).

At pH 3.0, 7.0, and 8.5, there was no significant effect of heating rate on the rigidity ($G'$) of 7% (w/v) EWP or WPI gels after 4 h at 80 °C (Table 3) and master curves normalizing based on gel time were detected (Figure 2). This shows that, at these pHs, all EWP and WPI gels produced by different heating rates eventually reached the same rigidity and that rigidity of gel networks for both proteins were clearly dependent on time allowed for an “equilibrium-like” state to be reached. Gel rigidities compared at the endpoint temperature were generally significantly different (Table 3), with slower heating rates producing more rigid gels. Li and others (2006) also observed a similar heating rate effect with 15% (w/v) WPI gels. It appears that the overall gel structure is fixed shortly after formation, while gel rigidity continued to increase due to addition of proteins to the network, or rearrangement of network strands, which strengthen the overall network (Verheul and Roefs 1998).

On the other hand, heating rate had an effect on the rigidity of WPI and EWP at pH 4.5. Gels formed by faster heating were more rigid than gels formed by slower heating (Table 3) and master curves based on normalized gel time were not detected for both proteins (Figure 2). At pH 4.5 where the predominant proteins in EWP and WPI are close to their pI's, intermolecular interactions started at room temperature (i.e., denaturation is not required). Therefore, heat-induced gelation became a competition between micro-phase separation and gelation. Gels produced by slower heating rates had lower rigidities than gels produced by faster heating rates due to more time for precipitation prior to gelation (Figure 3).

The gelation model of Ferry (1948) can be used to explain the effect of heating condition on gel formation (Figure 1). The model represents gelation as resulting from a native to denatured structural transition, represented by a 1st-order rate constant for denaturation ($k_d$) (Sanchez-Ruiz and others 1988) and $k_3$ as the rate constant for aggregation. If $k_3$ is slow on the time scale of the heating rate, then a rapid heating without subsequent holding prevents full network formation. Increased $G'$ after achieving 80 °C is clearly seen in all gelation conditions (Table 3). According to Ferry (1948), for a given rate of denaturation, the rate of aggregation will be slow if the attractive forces between the denatured protein chains are small. This results in a finer gel network with less opacity that exhibits less syneresis than the one with faster rate of aggregation (Ferry 1948). If different heating rates had no effect on the attractive forces among molecules, then the gel structure produced by different heating rates would be the same provide

<table>
<thead>
<tr>
<th>Table 3—Rheological properties of 7% (w/v) EWP and WPI gels.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>pH 3.0</strong></td>
</tr>
<tr>
<td><strong>G' (Pa) at endpoint temperature</strong></td>
</tr>
<tr>
<td>Whey protein isolate</td>
</tr>
<tr>
<td>20 °C/mm</td>
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<tr>
<td>0.2 °C/mm</td>
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<tr>
<td>Egg white protein</td>
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<tr>
<td>20 °C/mm</td>
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<tr>
<td>0.2 °C/mm</td>
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<td>2 °C/mm</td>
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</table>

Different letters in each column, among heating rates within protein type, are significantly different ($P < 0.05$) from each other.
sufficient time was allowed for aggregation into a gelled network. That appeared to be the case at pH 3.0, 7.0, and 8.5. However, when the system is in the micro-phase separated region (Ako and others 2009), the effect of heating rate cannot simply be predicted using diffusion limited conditions as proposed by Fougeler and others (1986). Moreover, at pH 4.5, medium heating rate (2 °C/min) produced the same gels as fast heating rate for EWP but not for WPI. This could be due to EWP being comprised of several proteins with a wide range of pHs. Furthermore, EWP had a lower gelation temperature than WPI, especially when the slower heating rate was used to form gels (Table 2). Tang and others (1994) also reported that EWP have a lower gelation temperature than WPC at a given concentration.

Mechanical spectra

After gels were formed by different heating rates and held for 4 h, the mechanical spectra were recorded at 80 °C. The mechanical spectra follow the same trends as the gelation process; no major effect of heating rate was observed at pH 3.0, 7.0, and 8.5, but an effect was seen at pH 4.5 (Figure 4). The mechanical spectra for treatments at pH 4.5 and 7.0 were prevented because pH 4.5 is in the micro-phase separate region and pH 7 represents single phase data; spectra seen at pH 3.0 or 8.5 were similar to the ones at pH 7.0.

The storage modulus of EWP gels prepared from different heating rates had a generally linear relationship over a range of 0.001 to 1 Hz at all pH levels, while the storage modulus of WPI shows a slight frequency dependence at a lower frequencies at pH 7.0 (Figure 4) and pH 8.5. The general frequency independence indicated that gels had a strong elastic component, whereas slight frequency dependence at low frequencies indicates some longer time network relaxation (Stefle 1996).

Phase separation—visual observations

Rheological properties only showed heating rate effects at pH 4.5. Visual observations and turbidity measurements were used to compare appearance. Protein concentrations were 1%, and 5% w/v to cover below and above critical gelation concentration of WPI at pH 7.0, respectively. The results of 5% (w/v) WPI gels (Figure 5) support the speculation on phase stability based on rheological measurements, as opaque gels were seen at pH 4.5. Phase separation was observed when gels were heated using slower heating rates, whereas the fast heating allowed for protein network formation prior to protein precipitation; thus, phase separation was not observed at the visual length scale (Figure 5). On the other hand, no phase separation was observed in all 5% (w/v) EWP gels (Figure 6).

The effects of heating rate on 1% (w/v) WPI and EWP solutions were investigated to determine if precipitation occurred below Cw. A precipitate was observed for WPI at pH 4.5; however, EWP still formed gels at pH 4.5 and 1% (w/v) protein (Figure 7 and 8). Turbidity increased as heating rate decreased for WPI solutions (Figure 7), as would be expected if particle size was increasing. In contrast, turbidity tended to decrease as heating rate decreased for EWP (Figure 8). The reasons for these opposite trends were not apparent, as turbidity is the result of a number of factors.

Phase separation observation by CLSM

The microstructure of 7% (w/v) WPI gels (Figure 9) fit the micro-phase separation model of Ako and others (2009). Only gels formed at pH 4.5 were heterogeneous at this length scale (above the resolution of this magnification; approximately 140 nm). In contrast, microstructures of EWP gels (Figure 10) were more complex. Gel structures at pH 3.0 were homogeneous but images at pH 7.0 and 8.5 showed some level of structure, where no structure was seen in WPI gels at similar pH values (Figure 9). EWP gels appeared to have a mild degree of micro-phase separation at all pH values from pH 4.5 to 8.5 but to a greater extent at pH 4.5. This coincides with the visual appearance of the gels (Figure 6). Moreover, for EWP, a separate feature of bigger aggregates were seen when slower heating rates were used at pH 4.5 (Figure 10), when compared to faster heating rates. Less microphase separation was observed at pH 4.5 with EWP than with
WPI (Figure 9 and 10). For WPI, aggregates on length scales of approximately 40 to 50 μm were seen at pH 4.5 for gels produced by all heating rates (Figure 9). However, the lack of clearly delineated aggregates prevents a quantitative determination of aggregate size and comparison among heating rates.

Changes in $G'$ showed that there was an effect of heating rate on the rigidity of WPI and EWP at pH 4.5: gels formed by faster heating were more rigid than gels formed by slower heating (Figure 2). For EWP, fast and medium heating rates produced significantly more rigid gels than slow heating rate at pH 4.5 (Table 3), and for WPI, gels those produced by the fastest rate were more rigid than those produced by medium and slow heating rates (Table 3). One plausible explanation for this difference is that the large aggregates produced in WPI (Figure 9) settle faster than EWP aggregates so only the fastest heating rate made a difference; however, more evidence is needed.

The difference in appearance of WPI and EWP gel microstructures could be due to protein composition. EWP is composed of several proteins including 54% ovalbumin that has pI of 4.5 and 3% to 12% of 6 additional proteins that have pIs ranging from 4.1 to 10.7 (Li-Chan and Nakai 1989). In comparison, WPI used in this study is composed of mainly 3 proteins; 65% β-lactoglobulin, 25% α-lactalbumin, and 8% bovine serum albumin and having an average pI of around 5.2 (Foegegin and Luck 2003). The range of pIs of proteins found in WPI and concentrations used in this study seems to cause less micro-phase separation than WPI at pH 4.5, but at the same time, some degree of micro-phase separation was observed over a wider pH range. In addition to differences in pIs among EWP's, some are known to be glycosylated and this may alter aggregation (Li-Chan and Nakai 1989).

Transition from straddle to micro-phase separate gels seen with light scattering and CLSM does not correspond to the transition between transparent and turbid gels (Ako and others 2009). At low ionic strength, the turbidity of the gels increased dramatically when the pH approaches the pI; this happens before micro-phase separation is observed. However, an increase in the turbidity of gels...
indicates that the gels are approaching the micro-phase separation region (Ako and others 2009). This agrees with our findings: WPI gels at pH 7.0 formed by slow heating rate seems to be very opaque but the microstructure was homogeneous when viewed under CLSM (Figure 5 and 9).

**Comparison with previous investigations**

It is interesting to review previous reports on heating rate effects and see if they can be explained based on limited heating time and/or being in the single phase or micro-phase separated regions. Stading and others (1992) investigated the effect of heating rate (0.017 to 12 °C/min to a 90 °C endpoint and a 60 min holding) on β-lactoglobulin (12%, 13%, and 14.6% w/w) gelation properties and microstructure in the single phase region at pH 7.5. Homogeneous microstructures were formed at fast heating rates and inhomogeneous at slow heating on the 5 to 150 min scale. They suggested that inhomogeneities formed during gelation are due to phase separation on the nanometer scale. Gels produced by fast heating had higher storage modulus than gels produced by slower heating. Our single phase results and those of Stading and others (1992) differ. One major difference among investigations is that their lowest heating rate requiring over 2 d to reach the final temperature. This may have allowed for settling of aggregates and a reduction in storage modulus.

The same group of researchers (Stading and others 1993) also investigated the effect of heating rate (0.1 to 12 °C/min to a 90 °C endpoint and a 60 min holding) on gelation of 10% (w/v) β-lactoglobulin at pH 5.3. β-Lactoglobulin has a pI of 5.2 (Swaisgood 1982); thus, at pH 5.3, it is considered to be in a 2-phase region. Networks formed by slow heating rates (0.1 to 1 °C/min) were inhomogeneous at a 500 to 2000 nm length scale, had larger pores, and the particle distribution size was also broader.
microstructural changes fit well with our observations on WPI gels at pH 4.5. However, gels formed at a slower heating rate had a higher G', while we found that the G' values were lower when slower heating rates were used in the phase separated region (Figure 2). The reason for this discrepancy is not clear, but it may be suggesting that there are differences within the phase-separated region (i.e., pH 4.5 and 5.3 are both micro-phase separated but form slightly different gel networks). One possible explanation for heating rate affects on rheological properties of gels formed in the micro-phase separate region is alteration of primary aggregates. Bromley and others (2006) reported that heating rate affects particle size and polydispersity of 3% w/w β-lactoglobulin at pH 5.3; particle size and polydispersity decrease as heating rate increases.

The effect of heating rate (1 to 5 °C/min to a 90 °C endpoint and a 60 min holding) on the pore size of particulate whey protein concentrate gels at pH 4.6 and pH 5.4 was also investigated by Langton and Herrnsson (1996). They found that the effect of heating rate on the pore size at pH 4.6 was not significant, but at pH 5.4, gels produced by slower heating rate had 7 times larger pore size than faster heating on a 100 to 2000 nm length scale. The larger pore size at the slower heating rate suggests more extensive phase separation. This result fits with their previous work at pH 5.3 (Stading and others 1993).

Insufficient time for full network formation can explain the results of several investigations. Foged and Others (1986) suggested that myosin and fibrinogen gels produced by faster heating rates were weaker than slower heating due to insufficient time for proteins to form a gel network. Arntfield and Murray (1992) looked at the effect of heating rate (0.3 to 3 °C/min to a common endpoint) on the rigidity and type of networks formed with ovalbumin and vicilin at different pHs (5.0, 7.0, and 9.0). When
proteins were in the single phase region, slower heating rates to a common endpoint temperature resulted in the most rigid networks but had little impact on the type of network on a 300 to 5000 nm length scale. This is logical due to insufficient time allowed for proteins to incorporate into the gel network and consistent with our results.

Conclusions

The effect of heating rate can be divided into 2 conditions. First, heating time sensitive gels form a common rheological endpoint, regardless of heating rate, when sufficient time is allowed for network formation. Second, heating time sensitive gels are those where variation in heating rate produces differences in rheological properties that remain after extended holding time. When gelation conditions are viewed based on the micro-phase separation model, it appears that heating rate sensitivity depends on if the gel is in the single phase or micro-phase separated regions. However, further validation of this model is needed to address inconsistencies with some previous investigation and account for protein concentration effects.

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References

CHAPTER 3
MANUSCRIPT 2

Effects of Heating Rate and pH on Fracture and Water-Holding Properties of Globular Protein Gels as Explained by Micro-Phase Separation

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Authors: Phanin N. Leksrisompong, Tyre C. Lanier and E. Allen Foegeding
ABSTRACT

The effect of heating rate and pH on fracture properties and held water (HW) of globular protein gels was investigated. The study was divided into two experiments. In the first experiment, whey protein isolate (WPI) and egg white protein (EWP) gels were formed at pH 4.5 and 7.0 using heating rates ranging from 0.1 to 35 °C/min and holding times at 80 °C up to 240 min. The second experiment used one heating condition (80 °C for 60 min) and probed in detail the pH range of 4.5 to 7.0 for EWP gels. Fracture properties of gels were measured by torsional deformation and HW was measured as the amount of fluid retained after a mild centrifugation. Single or micro-phase separated conditions were determined by confocal laser scanning microscopy. The effect of heating rate on fracture properties and HW of globular protein gels can be explained by phase stability of the protein dispersion and total thermal input. Minimal difference in fracture properties and HW of EWP gels at pH 4.5 versus 7.0 were observed while WPI gels were stronger and had higher HW at pH 7.0 as compared to 4.5. This was due to a mild degree of micro-phase separation of EWP gels across the pH range whereas WPI gels only showed an extreme micro-phase separation in a narrow pH range. In summary, gel formation and physical properties of globular protein gels can be explained by micro-phase separation.

Keywords: Egg white, Whey protein isolate, pH, Heating rate, Micro-phase separation
Practical Application:

The effect of heating conditions on hardness and water-holding properties of protein gels is explained by the relative percentage of micro-phase separated proteins. Heating rates that are too rapid require additional holding time at the end-point temperature to allow for full network development. Increase in degree of micro-phase separation decreases the ability for protein gels to hold water.
Introduction

A functional property of food proteins is the ability to form heat-induced gels. The gelation process depends on the balance between attractive and repulsive forces amongst the denatured protein molecules during aggregation. Varying solvent conditions (pH and ionic strength), protein type, concentration and processing parameters (heating temperature, time and rate) (Clark and Lee-Tuffnell 1986; Arntfield and others 1989; Langton and Hermansson 1992; Bowland and Foegeding 1995) affect this process by altering the way molecules organize and interact in the system. This in turn affects structures seen at the nano and micro levels, mechanical properties and ultimately, texture of food products. Of these parameters, heating rate and pH are of interest in this study.

Whey protein gels have been differentiated based on microscopic and macroscopic properties and characterized as fine-stranded or particulate gels. Fine-stranded gels have structures that are initially apparent on the nano-scale, are clear/translucent in appearance and tend to retain fluids when deformed. These gels appear to be homogenous at micro-length scale and are called single-phase based on the micro-phase separation model (Ako and others 2009). Particulate gels are opaque in appearance and release water during deformation (Langton and Hermansson 1992; van der Linden and Foegeding 2009). Formation of particulate gels is related to protein phase separation (Clark and others 1981); producing particles observed in the micro length scale - thus called micro-phase separation (Ako and others 2009). Moreover, fine-stranded whey protein gels formed at low (≤ 3.5) and high (≥ 6.5) pH have different textural properties; gels formed at low pH are weak and brittle,
whereas gels formed at high pH are strong and elastic (Tang and others 1995; Errington and Foegeding 1998).

Previously, the effect of heating rate on gelation and microstructure of egg white protein (EWP) and whey protein isolate (WPI) gels across the pH range of 3.0 to 8.5 was investigated (Leksrisompong and Foegeding 2011). The effect of heating rate on gel rigidity (storage modulus; G') can be explained based on phase stability and the kinetics of gelation versus precipitation. When electrostatic repulsion is high, the system is single phase and fine-stranded gels or soluble aggregates are formed depending on protein concentration (Ako and others 2009; Leksrisompong and Foegeding 2011). Under single phase conditions, there is no effect of heating rate on G' of gels, provided sufficient time is allowed for a completion of the aggregation process. In contrast, under micro-phase separated conditions, gel formation is a result of a competition between large aggregates settling or becoming frozen in the network prior to precipitation.

Rheological properties determined in the linear viscoelastic region, such as G', provide information on gel network formation and network structure but are not directly related to sensory properties. Fracture properties, fracture stress (σf; gel strength) and fracture strain (γf; deformation), and structural changes under large deformation, are related to inherent defects in the gel network (Luyten and others 1992) and are more related to sensorial texture, product quality and stability (Montejano and others 1985; van Vliet and Walstra 1995). Fracture properties show high correlation with the predicted sensory firmness of feta cheese (Wium and Qvist 1998), first bite and chew down sensory terms of agar/glycerol gels (Barrangou and others 2006b) and WPI/Kappa-carrageenan mixed gels.
(Çakır and others 2012) as well as several sensory terms of cheddar cheese (Everard and others 2006). In addition, the water holding/release properties of gels are important to overall sensory quality. Sensory analysis of WPI/gellan gum mixed gels shows the ability of gels to hold water is closely related to sensory perception of model foods (van den Berg and others 2007).

Besides altering the rigidity of gel structures, heating rate alters gel strength (Foegeding and others 1986; Stading and others 1993; Riemann and others 2004; Li and others 2006). Under single phase conditions (pH 7.0, heating rate 0.1 – 15 °C/min and a 0 min hold), $\sigma_f$ and $\gamma_f$ of WPI gels decrease and increase, respectively, with increased heating rate (Li and others 2006). Similar trends are observed with meat proteins, where slower heating rates produce stronger gels than faster heating rates when compared at a common end-point temperature (Foegeding and others 1986; Riemann and others 2004). However, if given enough time after reaching end-point temperature, gels prepared by rapid heating are as strong as gels prepared by slow heating (Riemann and others 2004). Alternatively, $\beta$-lactoglobulin gels have higher $\sigma_f$ and $\gamma_f$ when heating rate is increased under micro-phase separation conditions (pH 5.3, 1 and 12 °C/min and a 60 min hold) (Stading and others 1993). These results suggest a similar heating rate/displacement phase state mechanism controlling gel firmness ($G'$) and strength.

The objective of this study was to explain the effect of heating rate on the fracture and water-holding properties of EWP and WPI gels based on the protein micro-phase separation model (Ako and others 2009).
Materials and Methods

Materials

Spray dried EWP (82.5% protein, dry basis, N × 6.25) was donated from Michael Foods, Inc. (Minnetonka, MN) and stored at 4 ± 1 °C. Whey protein isolate (BiPro; 94.21% protein, dry basis, N × 6.38) was provided by Davisco Foods International, Inc. (Le Sueur, MN, U.S.A.) and stored at room temperature (22 ± 1 °C). Protein contents were determined by inductively coupled plasma atomic emission spectroscopy. Sodium hydroxide (ACS pellets) and sodium chloride were purchased from Fisher Scientific Inc. (Fair Lawn, NJ, U.S.A.). Rhodamine B was purchased from Invitrogen (Eugene, OR, U.S.A). All solutions were prepared with deionized water from a Dracor Water Systems (Durham, NC, U.S.A.) purification system and at a minimum resistivity of 18.2 MΩ-cm.

Sample preparation

Experiment 1 - Effect of heating rate on fracture and water-holding properties of EWP and WPI gels at pH 4.5 and 7.0.

Protein solutions

Protein solutions were prepared following the method of Leksrisompong and Foegeding (2011) with a slight modification. The pH of EWP and WPI solutions were adjusted to 4.5 or 7.0 using 1 N HCl or 1 N sodium hydroxide, respectively, prior to final adjustment to 15% (w/v) protein.
Gel formation

The protein solutions were heated in stainless steel tubes (19 mm diameter) which were coated with canola oil (PAM, ConAgra Foods, Omaha, NE, U.S.A.). The tubes were closed at the top and bottom with rubber stoppers to prevent evaporation.

Fast heating

Protein solutions (20 ± 1 °C) were heated in stainless steel tubes immersed in a water bath to an end point of 80 °C. Solutions at pH 7.0 were heated at 95 ± 1 °C; producing heating rates of 20 °C/min for EWP and 35 °C/min for WPI. Solutions at pH 4.5 were heated at 99 ± 1 °C; producing heating rates of 20 °C/min for EWP and 17 °C/min for WPI. The temperature was monitored using a thermocouple (Fisher Scientific, Pittsburgh, PA, U.S.A.). The differences between the heating rates obtained were due to factors regulating heat transfer, such as particle size. At pH 4.5, intermolecular interactions of WPI used in this study started at room temperature (i.e., denaturation is not required) and large aggregates were already observed at a visual length scale. Once 80 °C was reached, the tubes were transferred to a 80 ± 1 °C water bath and held for 0, 5, 10, 15, 20, 120, 240 min prior to rapid cooling by immersion in an ice water bath to decrease the temperature of the gels to 4 ± 1 °C at a rate of approximately 15 °C/min.

Medium and slow heating

Protein solutions in stainless steel tubes were heated from 20 ± 1 °C to 80 °C at 1 °C/min (medium) or 0.1 °C/min (slow) in a programmable water bath, followed by rapid cooling.
Experiment 2 – Effect of pH on fracture and water-holding properties of EWP gels

Protein solutions were prepared following the method of Leksrisompong and Foegeding (2011) with a slight modification. The pH of EWP and WPI solutions were adjusted to 4.5, 5.0, 5.25, 5.5, 5.75, 6.0, 6.5, and 7.0 prior to final adjustment to 10% (w/v) protein. One heating condition (80 °C for 60 min) was used for gel formation. Ten percent protein solutions were chosen because shell egg albumen contains approximately 10 to 11% protein (Li-Chan and Nakai 1989).

Large strain torsional testing

Gels were equilibrated to room temperature (22 ± 1 °C) and tested using the torsion method developed by Diehl and others (1979) as described by Kim and others (1986). Briefly, gels cylinders (19 mm diameter) were cut to 29 mm heights and notched plastic discs, 27 mm diameter by 1 mm thick (gel Consultants, Inc., Raleigh, N.C., U.S.A.), were glued to each end using instant adhesive cyanoacrylate glue (“Quicktite” Super Glue, Loctite Corp., Cleveland, Ohio, U.S.A.). The cylinders were mounted on a modified milling machine and ground into capstan shape with a center diameter of 10 mm, then mounted on the Hamann Torsion Gelometer (Model GC-TG92 US, Gel Consultants, Inc., Raleigh, N.C., U.S.A.). The samples were twisted at a strain rate of 0.26/s until fracture; torque and angular displacement at fracture were used to calculate $\sigma_f$ and $\gamma_f$ according to the method of Diehl and others (1979). Treatments were replicated three times with 6 to 8 samples tested per replicate.
**Held Water**

The held water of gels was measured using the microcentrifuge-base method of Kocher and Foegeding (1993). Each treatment was replicated three times with three gel samples tested per treatment. In short, gel cylinders (10 mm height x 4.3 mm diameter) were centrifuged at 153 x g for 10 min using a Beckman Microfuge 11 horizontal rotor microcentrifuge (Beckman Instruments Inc., Palo Alto, CA). Held water was calculated as the percent of total water remaining in the gel after centrifugation.

**Confocal laser scanning microscopy**

Gel microstructure was observed following the method of Leksrisompong and Foegeding (2011). One heating condition (80 ºC for 60 min) was used for gel formation.

**Statistical Analysis**

Data were analyzed with ANOVA using PROC GLM of SAS (version 9.2, SAS Institute Inc., Cary, NC) and comparisons of the means using Tukey’s significant difference test (p < 0.05).

**Results and Discussion**

**Experiment 1: Effect of heating rate on fracture and water-holding properties of EWP and WPI gels at pH 4.5 and 7.0**

**Fracture stress (σ_f)**

Without holding after reaching 80 ºC, EWP gels prepared at pH 7.0 by slow heating rates were stronger (higher σ_f) than those heated at faster rates (Table 1). Whey protein isolate gels prepared at a medium heating rate (1 ºC/min), were stronger than those from
slower or faster rates (Table 1). We cannot explain why this result was seen for WPI gels and it would require additional experiments to propose a plausible mechanism. This result was not due to protein hydrolysis during the slower heating because SDS-PAGE did not show any evidence of hydrolysis (data not shown). At pH 7.0, both protein gels prepared by the fast heating rate (EWP: 20 °C/min, WPI: 35 °C/min) and a 0 min hold time were the weakest (lowest $\sigma_f$) (Table 1). This was due to insufficient time allowed for complete gel formation since additional holding time increases $\sigma_f$. A 5 min hold at 80 °C was sufficient for proteins to form gel networks with strength similar or greater to gels formed at a medium heating rate (1 °C/min) (Table 1).

Gels formed at pH 4.5 were very different between protein types. For EWP, there were minimal differences seen between pH 4.5 and 7.0 (Table 1). In contrast, WPI gels formed at pH 4.5 were so weak they could not survive sample preparation for fracture testing until after a hold time of 2 hr (Table 1). Even then, they were half the gel strength of the weakest gel formed at pH 7.0. Gel networks were a result of a competition between phase-separated aggregates settling or becoming frozen in the network prior to precipitation. Therefore, when in the micro-phase separated region, a slow heating rate allowed more time for proteins to precipitate prior to gelation, preventing development of a strong gel network. Since WPI gels were extremely micro-phase separated and EWP gels were just slightly micro-phase separated at pH 4.5, the precipitation process was stronger in WPI gels (Leksrisompong and Foegeding 2011).

Overall, the effect of heating rate on $\sigma_f$ was more pronounced on WPI than on EWP gels (i.e., minor change in $\sigma_f$ of EWP gels during holding at pH 7.0). This is consistent with
WPI having slower gel network formation (i.e., slower development of G’) than EWP gels (Leksrisompong and Foegeding 2011) (Figure 1). Increases in gel strength could be due to an increase in the concentration density of protein strands (Vincent 1972) or the physical properties of individual protein strands (Woodward and Cotterill 1986). Therefore, it is compelling to ascribe these trends to a slower addition of proteins to WPI networks than EWP networks (e.g., slower network density development); however, changes within the formed network can also be contributing.

The formation of gels of lower strength prepared by rapid heating rates with no subsequent holding coincides with previous reports on various proteins (Foegeding and others 1986; Stading and others 1993; Riemann and others 2004; Li and others 2006). If initial aggregates remain dispersed (i.e., not precipitating), it appears to be a simple reaction time-limited effect and if total thermal input is equal, gelation proceeds to a similar result (Riemann and others 2004).

**Fracture strain (γ_f)**

Fracture strain reached a constant value when provided sufficient time for network formation (Table 1). However, the initial holding period produced a slight decrease in γ_f for WPI gels (pH 7.0 results, Table 1). A decrease in γ_f as polymer concentration increases has been observed for WPI (Foegeding 1992), β-lactoglobulin (Pouzot and others 2006), gelatin (Bot and others 1996) and agarose gels (Barrangou and others 2006a). Gels prepared with higher agarose concentrations are proposed to have a stronger network but less flexible
chains which rupture at lower deformation (McEvoy and others 1985; Barrangou and others 2006a).

Since the $\gamma_f$ of EWP and WPI are affected differently by holding time, the effect of heating rate on $\gamma_f$ cannot be explained based on diffusion limitations, as was done for $\sigma_f$ of protein gels. Fracture stress is controlled by a number of factors including the strand density, whereas $\gamma_f$ is controlled by the shape of the strands and the ability of strands to change form or extend during applied tension (Mellema and others 2002; Renkema 2004). This implies: 1) At pH 7.0, the strands in EWP gels are fixed at a low extent of aggregation as compared to WPI gels and 2) an increase in strand network density causes the WPI gel network to become less deformable. However, further investigation is needed in order to make a valid conclusion.

**Water-Holding Properties**

Held water (HW) was measured as the amount of water retained after a mild centrifugation (designed for minimal structural breakdown of the gel network). There was no major effect of heating rate on HW for both proteins; however, there were differences among the treatments in relative magnitude (Table 2). It would appear that the gel network microstructure, which is highly dependent on solvent quality (Woodward and Cotterill 1986; Handa and others 1998; Çakır and others 2012) and regulates water movement (van den Berg and others 2007), was fixed at the initial stage of gelation and did not change significantly with holding time. It has been shown that gel network microstructure and permeability are established at the gelation point. Addition of protein molecules to the network post gel point
increases gel firmness but does not change water permeability (Verheul and Roefs 1998). Our results showing an increase in $\sigma_f$ with holding time (Table 1) but no change in HW (Table 2) supports this basic model.

**Overall Effects of Heating Rate**

The fracture properties together with HW results suggest that the effect of heating rate is dependent on two factors. In single phase conditions (pH 7.0 in this case), the initial gel network determines $\gamma_f$ and HW and is independent of heating rate. Once the network is formed, additional time is needed to fully develop gel strength (Table 1) and gel firmness ($G'$) (Leksrisompong and Foegeding 2011). Under micro-phase separation conditions, the heating rate is critical to gel formation because gelation is a competition between precipitation and locking particles into a gel network. However, once a gel is formed, the effects of holding time are similar to those observed for single phase systems.

In addition to heating rate, it is important to note that the time between the temperature where the aggregation rate becomes significant (Ts) and the final temperature is another important parameter. This temperature, Ts, depends on the pH and the type of protein.

**Experiment 2: Effect of pH on fracture and water-holding properties of EWP gels.**

Fracture properties and HW of WPI gels were lower at pH 4.5 compared to pH 7.0 (Table 1 and 2). Previous researchers (Verhuel and Roefs 1998; Barden and others 2010; Çakir and Foegeding 2011) also reported low fracture properties and HW of WPI gels.
formed under conditions where the electrostatic stabilization is low (i.e., pH close to pI or high ionic strength) and particulate (micro-phase separated) gels are formed. Under these conditions, WPI aggregates are formed prior to extensive protein unfolding and large primary aggregates undergo secondary aggregation to produce particulate gels (Langton and Hermansson 1992; Verhuel and Roefs 1998) with low HW and greater moisture permeability due to coarsening of the microstructure and an increase in voids (Verhuel and Roefs 1998). In contrast, fracture properties and HW of EWP gels at pH 4.5 and 7.0 were fairly similar (Tables 1 and 2). The $\sigma_f$ results follow the same trend as our previous findings, where the rigidity ($G'$) was higher at pH 4.5 than 7.0 for EWP and the opposite was true for WPI (Leksrisompong and Foegeding 2011).

To further investigate the pH effect on gels, the degree of micro-phase separation (Figure 2) and gelation properties (Figure 3) of 10% (w/v) EWP were observed over the range of pH 4.5 to 7.0. Some degree of micro-phase separation was observed over the entire pH range but was more extreme at pH 5.0 to 6.0 (Figure 2). A varying degree of micro-phase separation is consistent with egg white containing proteins that have pIs ranging from 3.9 to 10.7 (Li-Chan and Nakai 1989), and therefore, at any pH over this range there will be some protein in the micro-phase separated state. Scanning electron microscopy images of EWP gels at pH 5.0 and 6.0 also revealed large aggregate structures (Figure 4). In comparison, WPI showed an extreme degree of micro-phase separation at pH 4.5 (Figure 5). This agrees with the results of this investigation; and is consistent with WPI being comprised of mainly two proteins having average pI around 5.2 (Foegeding and Luck 2003). The results suggest that the differences in pH effects on fracture properties are due to protein
composition. In addition, EWP is comprised of 54% ovalbumin which has a pI of 4.5, but the most micro-phase separated structure does not occur at pH 4.5 (Figure 2). Similarly, β-lactoglobulin is the main protein (65%) in WPI and has a pI of 5.2, but the most micro-phase separated structure occurs at pH 4.5 (Lekrisompong and Foegeding 2011; Çakır 2011) (Figure 4 and 5). It is not surprising that net charge does not comprehensively explain the interactions among proteins. Charge distribution, as well as other types of interactions, will also contribute to aggregation. More work is needed to explain the cause of micro-phase separation in protein ingredient gels.

The structure at micro length scale correlates well with HW; EWP gels prepared in the pH range showing the greatest degree of micro-phase separation (pH 5.0 to 6.0) (Figure 2) have lower HW (Figure 3b). Moving away from this region, EWP gels became less micro-phase separated and HW increases (Figure 2 and 3b). These results follow the same trend as reported by previous researchers (van den Berg and others 2007; Çakır and Foegeding 2011) when WPI gels were investigated; homogenous structure gels had high HW, whereas micro-phase separated gels had lower HW. This explains why there was no significant difference between HW of EWP gels prepared at pH 4.5 and 7.0.

Macroscopic properties of EWP gels across the pH range of 4.5 to 9.0 reported by previous researchers (Beveridge and others 1980; Woodward and Cotterill 1986; Handa and others 1998; Lekrisompong and Foegeding 2011) were normalized and compared with our findings. Egg white protein gels were stronger at pH 4.5 to 6.0 and weakest at approximately pH 7.0 (Figure 6a). The trend of normalized HW (Figure 6b) showed no difference in the ability of EWP gels to hold water at pH 4.5 versus pH 7.0, and HW was lowest at pH 5.0 to
6.0. Gel strength and HW increase from pH 7.0 to 9.0 (Figure 6). A comparison of the effect of pH on $\sigma_f$ of WPI and EWP gels is seen in Figure 3a. At pH 6.0 and above, $\sigma_f$ decreases for EWP and increases for WPI. While the reason for this trend cannot be established, it highlights the potential role of ovotransferin, avidin and lysozyme, with a pI of 6.0, 10.0 and 10.7, respectively (Li-Chan and Nakai 1989), in gelation at high pH.

Conclusions

The protein micro-phase separation model of Ako and others (2009) can be used to explain fracture and water-holding properties of globular proteins gels. Based on this model, solvent quality, as related to electrostatic stabilization, establishes the basic structure of globular protein gels by determining which proteins, and to what extent, they can be considered micro-phase separated. Micro-phase separated proteins aggregate rapidly so gelation is a competition between phase separation and forming a gel network. The situation is more complex in mixed protein systems where each protein has the potential to be micro-phase separated or single phase. This explains why physical properties of EWP gels are less affected by pH in this region.

Acknowledgements

Support from the North Carolina Agricultural Research Service and American Egg Board are gratefully acknowledged. The use of trade names in this publication does not imply endorsement by the North Carolina Agricultural Research Service of the products named nor criticism of similar ones not mentioned. The authors are very grateful for the egg
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REFERENCES


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<th>EWP pH 7.0</th>
<th>WPI pH 4.5</th>
<th>WPI pH 7.0</th>
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Different letters in each column are significantly different (p <0.05) from each other.

*20°C/min for EWP, 17°C/min and 35°C/min WPI pH 4.5 and 7.0, respectively
** 1°C/min
*** 0.1°C/min

“–” indicates missing data points were due to:
1) Insufficient time for gelation (WPI: fast heating rate and 0 min hold),
2) Brittle gels that fractured prior to testing (WPI: fast heating rate and a 5 - 20 min hold) and
3) Inhomogeneous along the height of the samples due to phase separation (WPI: medium and slow heating rate, EWP: slow heating rate)
Table 2. Effect of heating rate and holding time on held water of 15% (w/v) EWP and WPI gels at pH 4.5 and 7.0.

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** 1˚C/min
*** 0.1˚C/min
“–” indicates missing data points were due to:
1) Insufficient time for gelation (WPI: fast heating rate and 0 min hold),
2) Inhomogeneous along the height of the samples due to phase separation (WPI: medium and slow heating rate, EWP: slow heating rate)
Figure 1. The effect of holding time after reaching 80 °C on fracture stress (15% w/v; ○) and G' (7% w/v; □) of EWP and WPI gels. (A), EWP pH 4.5; (B), EWP pH 7.0; (C), WPI pH 7.0.
Figure 2. Microstructure of 10% (w/v) EWP gels (heated at 80 ºC for 60 min) at pH: A. 4.5; B. 5.0; C. 5.25; D. 5.5; E. 5.75; F. 6.0; G. 6.5; H. 7.0. Image size: 70.7 x 70.7 μm.
Figure 3. The effect of pH (4.5 – 7.0) on fracture properties and held-water of gels. (A), fracture stress (left axis) of 10% (w/v) EWP (□) and 10% (w/v) WPI (♦) gels (Foegeding 1993); and fracture stress (right axis) of gels from experiment 1: 15% (w/v) EWP (Δ) and WPI (▲); (B), fracture strain (□) and % HW (●) of 10% (w/v) EWP gels.
Figure 4. Structure of EWP gels at different length scales at pH 3.0 to 9.0. Top and middle rows are respective transmission and scanning electron microscopy images used with permission from Handa and others (1998) (bar is 100 nm). Bottom row is from Leksrisompong and Foegeding (2011) (image size: 70.7 x 70.7 μm) with permission. Gel images are centered over the gelling solution pH.
Figure 5. Structure of WPI gels at pH 3 to 8.5. Top and middle rows are from Çakir (2011) (image size: 212 x 212 μm) and the bottom row is from Leksrisompong and Foegeding 2011 (image size is 70.7 x 70.7 μm) with permission. Gel images are centered over the gelling solution pH.
Figure 6. Trend in normalized (relative to pH 7.0) strength (hardness, force, or $\sigma_f$) (A) and held water (B) of EWP gels across pH. Data are from Woodward and Cotterill 1986 (□); Handa and others 1998 (○); Beveridge and others 1980 (Δ); experiment 1- fast heating rate and a 20 min hold (◆); and experiment 2 (▼).
CHAPTER 4
MANUSCRIPT 3

Gelation Properties of Egg White Protein/Polysaccharide Mixed Gels Based on Micro-
Phase Separation

Manuscript to be submitted for publication:

Food Hydrocolloids

Authors: Phanin N. Leksrisompong and E. Allen Foegeding
Graphical abstract

Polysaccharide concentration

Keys:  Held water;  Recoverable energy;  Fracture stress
Abstract

This study investigated the microstructure and gelation properties of egg white protein (EWP)/polysaccharide mixed gels. Protein (10 % w/v protein; pH 7.0) and polysaccharides (0 to 0.6% w/w; guar gum, locust bean gum, high methoxyl pectin, κ-carrageenan, low methoxyl pectin, and ι-carrageenan) solutions were used to prepare mixed gels. Altering polysaccharide type and concentration produces a wide range of microstructures, as determined by confocal laser scanning microscopy, and physical properties of the mixed gels. Changes in fracture and water holding properties of gels corresponded to changes in microstructure, to a limited degree. The effect of polysaccharides type and concentration on these properties of the mixed gels can be grouped based on the charge density of the polysaccharide. The combination of polysaccharide charge density and micro-phase separation state of proteins was shown to be the key to predict the microstructure of mixed gels.

Keywords: egg white protein; protein-polysaccharide interactions; micro-phase separation; polysaccharide charge density; gelation properties
1. Introduction

Consumer trends are constantly changing and the demand for healthy, natural and convenient food products are on the rise. As a result, product developers are continuously challenged to design innovative products in a short period of time. Changing composition often alter food structure which results in changing the rheological properties and ultimately textural properties of food products (Renard, van de Veldve, & Visschers, 2006). Texture is one of the most important sensory attributes determining quality and consumer acceptability of foods (Wilkinson, Dijksterhuis, & Minekus, 2000). Other than sensory properties, food texture has been shown to impact human health (Norton & Norton, 2010). A good understanding of food structure is the key to design specific texture as well as to be able to alter composition of food products to meet the consumer demand without decrease their acceptability (Renard, van de Veldve, & Visschers, 2006; Norton & Norton, 2010).

It is well known that altering solvent conditions creates different structures of whey protein (WP) gels (Clark, Judge, Richards, Stubbs, & Suggett, 1981; Langton & Hermansson, 1992; McGuffey & Foegeding, 2001). Based on electron microscopy images of gel networks, fine-stranded gels are produced under conditions that favor electrostatic repulsions (pH away from isoelectric point (pl) and low ionic strength), whereas particulate gels are produced under conditions that favor electrostatic interactions (Langton & Hermansson, 1992). Recently, Ako, Nicolai, Durand and Brotons (2009) proposed a new way to aggregate and gel structure based on protein phase stability. Protein solutions are categorized as being single phase or two phase based on solvent conditions. There is a pH range where the system moves from single phase to two phase region as the system moves towards the
isoelectric point (pI) of the protein. When heating at concentrations below the critical
gelation concentration ($C_o$), the aggregates will remain soluble under single phase region and
precipitates will formed under two phase region. Whereas, at concentrations above $C_o$, gel
structure appears at the micrometer scale as homogenous under single phase region and
micro-phase separated structure is seen under two phase region (Ako et al., 2009).

Biopolymer mixtures can be use to alter food structure (Nishinari, Zhang, & Ikeda, 2000). When protein and polysaccharide are mixed with water there are several possible outcomes. At low concentrations, both biopolymers are soluble and form a mixed solution. At higher concentrations there is the possibility of associative (interactions between biopolymers) or segregative (no interactions between biopolymers) types of interactions (Tolstoguzov, 1991). This means that when concentrations are high enough for gelation, three types of mixed gels can be formed depending on the biopolymer’s molecular properties (i.e., shape, size, and charge) and solvent qualities (i.e., pH and ionic type/strength): interpenetrating, associative phase separated and segregative phase separated networks (Tolstoguzov, 1991; Piculell & Lindman, 1992; Turgeon, Beaulieu, Schmitt, & Sanchez, 2003). Interpenetrating networks are observed when the two components form continuous independent networks (Morris, 1986). However, mixtures of biopolymers often result in attractive or repulsive forces which lead to the formation of biopolymer associative and segregative phase separated networks, respectively (Morris, 1986; Tolstoguzov, 1991). Associative phase separation is due to intermolecular interactions between different biopolymers, forming complexes resulting in a structured phase that contains both types of biopolymers. Segregative phase separation, in contrast, is caused by thermodynamic
incompatibility of the biopolymers, resulting in a phase separated system; one phase rich in polysaccharides and the other rich in proteins separates by solvent (Grinberg & Tolstoguzov, 1997). However, it is should be noted that protein/polysaccharide models are based on mixture of two polymers whereas foods are more complex since they often contain more than two biopolymers.

Formation and properties of whey protein/polysaccharide mixed gels has been investigated (Sanchez, Schmitt, Babak, & Hardy, 1997; Beaulieu, Turgeon, & Doublier, 2001; van den Berg, van Vliet, van der Linden, van Boekel, & van de Velde, 2007a; de Jong & van de Velde, 2007; de Jong, Klok, & van de Velde, 2009; Çakır & Foegeding, 2011). Altering polysaccharide type and concentration produces a wide range of microstructure of whey protein isolate (WPI)/polysaccharide mixed gels. The microstructure of mixed gels has been characterized as “homogenous”, micro-phase separated (“protein continuous”, “bicontinuous”, and “coarse stranded”) and “polysaccharide continuous” (van den Berg et al., 2007a). “Homogenous” and “protein continuous” structures were shown to have higher water holding properties than “bicontinuous” and “coarse stranded” gels (van den Berg et al., 2007a; Çakır & Foegeding, 2011). Molecular properties of polysaccharides, mainly charge density and polysaccharide concentration, determined the microstructure, and in turn fracture properties of WPI/polysaccharide mixed gels (de Jong & van de Velde, 2007). Polysaccharides can be divided into three categories; low, medium and high charge density, based on how they alter gel structure (de Jong & van de Velde, 2007). Low charge density polysaccharides formed “protein continuous” and “bicontinuous” microstructures. Medium charge density form “coarse stranded” microstructure and high charge density form
“homogenous” microstructure. Solvent quality (i.e., pH and ionic strength) also has significant effect on gel structure (Çakır & Foegeding, 2011; Çakır, 2011). In the single phase region of whey proteins (pH 7.0 and 50 mM NaCl), structure changes from “homogenous” to “protein continuous” to “bicontinuous” and finally “phase inversion” as κ-carrageenan concentration increases from 0 to 0.6% (w/w) (Çakır & Foegeding, 2011). In the two phase region (pH 5.5 and 100 mM NaCl or pH 7.0 and 250 mM NaCl), the structure change from “particulate” to “coarse stranded” to “polysaccharide continuous” structures (Çakır, 2011). Changes in microstructures have been associated with specific sensory properties of mixed gels (van den Berg et al., 2007a; de Jong & van de Velde, 2007; Çakır & Foegeding, 2011).

To our knowledge, heat-induced gelation of egg white protein (EWP)/polysaccharide mixed gels had not been studied thus far. Egg white protein and WP are used extensively as ingredients in the food industry. Despite their similarity (i.e., globular proteins), they were shown to have different structures, gel formation and gelation properties (Leksrisompong & Foegeding, 2011; Leksrisompong, Lanier, & Foegeding, Submitted). Whey protein isolate gels showed an extreme degree of micro-phase separation at a pH close to their pI. In addition, gel strength and water holding properties were lowest close to this region. On the other hand, EWP gels show a varying degree of micro-phase separation and a minimal change in the water holding properties across the pH range (pH 3.0 to 8.5). Combined mechanisms of micro-phase separation model (Ako et al., 2009) with polysaccharide charge density model (de Jong & van de Velde, 2007) will lead to a better understanding of EWP/polysaccharide interactions. The objective of this study is to investigate the effect of
polysaccharide type and concentration on the microstructure and gelation properties of EWP/polysaccharide mixed gels.

2. Materials and methods

2.1. Materials

Spray dried EWP (82.5% protein, dry basis) was provided by Michael Foods, Inc. (Minnetonka, MN, USA) and stored at 4 ± 1°C. The mineral content was 0.11% P, 0.8% Ca, 0.075% Mg, 0.9% K, 1.4% S and 0.012% Na). Protein and mineral contents were determined by inductively coupled plasma atomic emission spectroscopy. Guar gum (GRINDSTED GUAR 5000), high methoxyl pectin (GRINDSTED PECTIN RS 461; degree of esterification (DE)); low methoxyl pectin (GRINDSTED PECTIN LC 950; DE 32%), and κ-carrageenan (GRINDSTED CX 302) were donated by Danisco (New Century, KS, USA). Locust bean gum (GENU GUM RL-200Z) and κ- carrageenan (GENUGEL CHP-2) were kindly provided by CP Kelco Inc. (Lille Skensved, Denmark). Sodium hydroxide (ACS pellets) was purchased from Fisher Scientific Inc. (Fair Lawn, NJ, USA). Rhodamine B was purchased from Invitrogen (Eugene, OR, USA). All materials were used without further purification. Deionized water was obtained using a Dracor Water Systems (Durham, NC, USA) purification system to produce a minimum resistivity of 18.2 MΩ-cm.

2.2. Gel preparation

2.2.1. Protein and polysaccharide stock solutions preparation

Egg white protein solutions were prepared following the method of Leksrisomponng and Foegeding (2011), with a slight modification. Egg white protein solutions were prepared by hydrating in deionized water with continuous stirring for approximately 8 h. The pH of
EWP solutions was adjusted to 7.0 using 1 N sodium hydroxide prior to final adjustment to 20% (w/v) protein (double concentration is required to achieved final concentration). Polysaccharides (0.2, 0.4, 0.6, 0.8, 1.0, and 1.2% w/w) were dissolved in deionized water by first stirring over night at room temperature (22 ± 2 ºC) and then heating at 80 ºC in a water bath for 30 min to allow for full hydration.

2.2.2. Protein and polysaccharide mixture

Prior to mixing protein and polysaccharide stock solutions were heated at 45 ºC water bath for approximately 15 min to allow temperature equilibration. This temperature was high enough to prevent polysaccharide gelation as well as below the denaturation temperature of EWP. The stock solutions of protein and polysaccharides were then mixed at a 1:1 ratio. Egg white protein gels were prepared as above except deionized water was used for the dilution.

2.2.3. Gel formation for rheological measurement

Solution mixtures were poured into glass tubes (diameter 19 mm) and heated in an 80 ºC water bath for 30 min followed by cooling at room temperature (22 ± 1 ºC) for 2 h and refrigeration overnight at 4 ± 1 ºC. Gels were equilibrated to room temperature (22 ± 1 ºC) prior to all tests.

2.2.4. Gel formation for microstructure observation

Gel formation for microstructure observation was performed following the method of Leksisrompong and Foegeding (2011), with slight modification. Protein and polysaccharide mixtures were labeled with Rhdamine B (10 μL of a 0.2% (w/w) Rhodamine B + 1 mL of mixtures) prior to pipetting into glass microwell dishes (MatTek corporation, MA, USA) and
sealed. The mixture was heated in an 80 °C water bath for 30 min followed by cooling at room temperature (22 ± 1 °C) for 2 h and refrigeration overnight at 4 ± 1 °C.

2.3. Methods

2.3.1. Uniaxial compression

Gel samples were cut into cylinders of 21.5 mm height and 19 mm diameter. Uniaxial compression measurements conducted using an Instron universal testing instrument, type 5565 (Instron engineering Corp., Canton, MA, USA) equipped with a 5 kN load cell. A thin coating of mineral oil was applied to the top and bottom of the plate to prevent friction during compression.

2.3.1.1. Percent Recoverable Energy

The percent recoverable energy (RE) was determined following the method of van den Berg, Carolas, van Vliet, van der Linden, van Boekel, and van de Velde (2008). Gels were compressed at a constant compression speed of 50 mm/min, to 75% of the initial height (to avoid macroscopic fracture) and decompressed at the same rate, allowing recovery. Percent RE was calculated from the force-deformation curve as a ratio of work recovered from decompression (area under the second half of the curve) divided by the total work to compress (area under the first half of the curve). Recoverable and irrecoverable work represents the contribution of stored and dissipated energy, respectively (Nussinovitch, Kaletunc, Normad, & Peleg, 1990).

2.3.1.2. Large deformation rheology

Fracture properties were performed by compressing the samples at a rate of 50 mm/min to 20% of the initial height. True stress (σT) and True (Hencky’s) strain (εH) were
calculated at every time point throughout the measurement using the equation of Truong and Daubert (2000). Breakdown patterns of EWP gels and EWP/polysaccharide mixed gels were obtained from compression tests by normalizing the force and deformation curve by the fracture points following the method of van den Berg et al., 2008.

2.3.2. Held water

The held water (HW) of gels was measured using the microcentrifuge-based method of Kocher and Foegeding (1993). Gel cylinders (10 mm height x 4.3 mm diameter) were placed in microcentrifuge filtration tubes (2.0 mL) which allowed the collection of released fluid when subjected to a mild centrifugation. The samples were centrifuge at 153 g for 10 min using a VWR Symphony 2417R microcentrifuge (VWR International, Radnor, PA, USA). The total moisture content of gels (w/w) was determined by drying the samples in a 100 °C oven for 12 h. Held water was calculated as the percent of total water remaining in the gel after centrifugation. Each treatment was replicated three times with three gel samples tested per treatment.

2.3.3. Confocal Laser Scanning Microscope (CLSM)

Gel microstructure was observed using a Zeiss LSM 710 CLSM equipped with an inverted microscope (Zeiss Axio Observer Z1) in the fluorescence mode. The objective lens used was a 40x (LD C-Apochromat 40x/1.1 W Korr M27). The excitation was at 514 nm and the emission of Rhodamine B was recorded between 546 to 700 nm.
2.3.4. **Statistical analysis**

Data were analyzed using ANOVA using PROC GLM of SAS (version 9.2, SAS Institute Inc., Cary, NC, USA) and comparisons of the means using Tukey's significant difference test (p < 0.05).

3. **Results**

3.1. **Microstructure and gelation properties of heat-induced EWP gels**

Fig. 1 shows microstructure of 10% (w/v) heat-induced EWP gel at pH 7.0. The bright areas represent protein (stained by Rhodamine B) and the dark areas represent zones devoid of proteins which are mainly comprised of water. A mild degree of micro-phase separation observed at pH 7.0 (Fig. 1A) agrees with previous work (Leksrisompong & Foegeding, 2011; Leksrisompong et al., Submitted). Held water is the amount of water retained by the protein network after a mild centrifugation and the percent recoverable energy (RE) represents the amount of energy stored by the material after 25% compression. Heat-induced EWP gels at pH 7.0 had 63% HW and 37% RE (Fig. 3 to 7). Fracture stress ($\sigma_f$) and fracture strain ($\gamma_f$) represent strength and deformability of the gels, respectively. The fracture properties reveal that EWP formed a relatively weak gel ($\sigma_f = 6.8$ kPa; $\gamma_f = 0.75$) at pH 7.0; which agrees with previous reports (Handa, Takahashi, Kuroda, & Froning, 1998; Leksrisompong et al., Submitted). The breakdown pattern after fracture point is related to elastically stored energy and macroscopic breakdown of the material (van den Berg et al., 2008). The slope of the EWP gel breakdown pattern shows a semi-steep decrease which is similar to the breakdown pattern of particulate WPI gels (Çakır, Daubert, Drake, Vinyard, Essick, & Foegeding, 2012).
3.2. Microstructure and gelation properties of EWP/polysaccharide mixed gels

The microstructures of EWP (10% w/v, pH 7.0) and polysaccharides (0 to 0.6% w/w) mixed gels are shown in Fig. 1. Overall, EWP/polysaccharide mixed gels showed phase separated structure except for EWP/LM pectin mixed gels. In the case of mixed gels, the dark areas represent the area devoid of protein which is known as “serum phase” comprised of mostly water and polysaccharide. Based on the charge density model of de Jong and van de Velde (2007), the effect of polysaccharides concentration on microstructure and gelation properties of EWP/polysaccharide mixed gels can be grouped into 4 categories: neutral, low, medium and high charge density (Charge density of 0, 0.29, 0.5 and > 0.63 mol negative charge/mol monosaccharide, respectively). We investigated neutral (guar gum and locust bean gum (LBG)), low charge density (HM pectin), medium (κ-carrageenan) and high (LM pectin and ι-carrageenan) charge density polysaccharide.

3.2.1. Neutral: Guar gum and locust bean gum

The first category includes guar and LBG known as the galactomannans. Addition of only 0.2% (w/w) of guar gum and LBG resulted in phase inversion where the polysaccharides became the continuous phase and proteins were compressed into concentrated droplets (Fig. 1; the first and the second column and Fig. 2). However, the phase inversion structure was not as extreme and not as distinct as cold set WPI/galactomannan mixed gels observed by de Jong and van de Velde (2007).
An attempt was made to measure HW of the mixed gels, however, accurate results were not possible because small gel pieces fell to the bottom of the microcentrifuge tubes after a mild centrifugation. There was no significant difference between the RE of the EWP gels and EWP/galactomannan mixed gels (0.1% w/w) (Fig. 3B).

As the concentration increased from 0 to 0.1% (w/w), a slight decrease in gel strength was observed. The samples were not strong enough for mechanical testing at or above 0.2% (w/w) galactomannans (Fig. 3A). The slope of the breakdown pattern of the EWP/galactomannan mixed gel (0.1% w/w) was more gradual when compared to EWP gel (Fig. 3C).

3.2.2. Low charge density: High methoxyl pectin

The degree of micro-phase separation of EWP/HM pectin mixed gels increased slightly as concentration of HM pectin increased; proteins formed larger aggregates resulting in coarsening of microstructure (Fig. 1; the third column). This was associated with slight decrease in HW and RE (Fig. 4B). There were also slight decreased in fracture stress and increased in fracture strain as the microstructure became coarser (Fig. 1 and 4A). The breakdown patterns of EWP/HM pectin gels showed interesting trends (Fig. 4C). Increased concentration of polysaccharide resulted in a more gradual breakdown path and eventually a second hardening phase. The slope of the breakdown pattern has been shown to flatten as the microstructure of the gels shift from particulate to coarse stranded (Çakır et al., 2012).

3.2.3. Medium charge density: κ-carrageenan

The effect of κ-carrageenan on microstructure and rheological properties of mixed gels was highly concentration dependent. As κ-carrageenan concentration increased from 0.0
to 0.4% (w/w), the change in microstructure of the mixed gel was similar to addition of HM and LM pectin (somewhere in between). Above 0.4% (w/w), the microstructure became coarser (Fig. 1; the fourth column). Phase separation forced the protein phase to be concentrated as the area of the serum phase was increased (Fig. 1).

As the concentration of κ-carrageenan in the mixed gels increased, the HW also increased (Fig. 5B). This trend was also observed with heat-induced WPI/κ-carrageenan mixed gels as long as the proteins remain the continuous phase (Çakır & Foegeding, 2011). However, addition of κ-carrageenan showed minimal change in the RE of the mixed gels (Fig. 5B). Increasing the κ-carrageenan concentration resulted in an increase in σ_f and a decrease in γ_f (Fig. 5A). Egg white protein/κ-carrageenan mixed gels showed the highest variation in fracture properties; the σ_f varied from 6.8 to 27 kPa and γ_f varied from 0.35 to 0.79. The mixed gels became more brittle (i.e., lower γ_f) as the concentration increased. The effect of κ-carrageenan concentration on the fracture properties and HW of the EWP/κ-carrageenan mixed gels is similar to the effect of increasing protein concentration of WPI gels (Foegeding, 1992; Errington & Foegeding, 1998). The breakdown pattern slope became slightly more gradual when κ-carrageenan was added, but no clear trend in effect of κ-carrageenan concentration on the breakdown pattern was seen (Fig. 5C).

3.2.4. High charge density: Low methoxyl pectin and ι-carrageenan

Low methoxyl (LM) pectin and ι-carrageenan are categorized as high charge density polysaccharides. Unlike HM pectin, addition of LM pectin had no impact on the microstructure of the mixed gels at this length scale; microstructures of EWP/LM pectin mixed gels were comparable with that of EWP gels (Fig. 1; the fifth column). This trend was
also observed with acid-induced WPI/LM pectin mixed gels. An increase in the degree of coarseness of acid induced WPI/pectin mixed gels had been shown to depend on both the pectin concentration and the degree of esterification (i.e., high degree of esterification resulted in coarser structure) (de Jong & van de Velde, 2007). An interesting structure was observed when ι-carrageenan was added; the presence of ι-carrageenan coarsen the microstructure, although ι-carrageenan concentration had minimal impact on the microstructure (Fig. 1; the sixth column). This was not the observed in cold set WPI/ι-carrageenan mixed gels; where ι-carrageenan had no effect on the gel microstructure (de Jong & van de Velde, 2007).

Increasing in LM pectin and ι-carrageenan concentration resulted in no change in RE but the HW increased (Fig. 6B and 7B), similar to the trend observed with EWP/κ-carrageenan mixed gels. Addition of LM pectin and ι-carrageenan resulted in similar trends in fracture and breakdown properties (Fig. 6 and 7).

4. Discussion

4.1. Effect of polysaccharide type and concentration on microstructure of mixed gels

At pH 7.0, EWP gels showed a mild degree of micro-phase separation (Fig. 1; the first row). Since EWP contains a mix of proteins that have pIs ranging from 3.9 to 10.7 (Li-Chan & Nakai, 1989), one would expect some visible particles resulting from individual proteins being in their micro-phase separated state at this pH. Heat induced EWP/polysaccharide mixed gels also resulted in micro-phase separated structures (Fig. 1). However, the structures were different from protein micro-phase separation since the final microstructure of mixed gels is a result of a competition between gel formation and the phase
separation processes between proteins and proteins/polysaccharides. Similar to previously reported by de Jong and van de Velde (2007), polysaccharide charge density and concentration are important factors determined the type of microstructure formed. In addition, charge density of the proteins (pH) also needs to be considered (Çakır, 2011). These properties dictate the affinity of the biopolymers towards each other or towards themselves prior to gelation.

At low polysaccharide concentrations, addition of lower charge density polysaccharides resulted in a higher degree of micro-phase separation than when higher charge density polysaccharides were added with exception of ι-carrageenan (Fig. 1; the second row). Similar trends were observed by de Jong and van de Velde (2007) (Fig. 8). Concentration of neutral, low, and medium charge density polysaccharides (categories 1 to 3) also affected the degree of micro-phase separation and dictated whether the protein or polysaccharide would constitute the continuous phase (Fig.1; the first to the fourth column). Whereas, increasing concentration of high charge density polysaccharides resulted in no change in the microstructures of mixed gels (Fig. 1; the fifth and the sixth column).

Microstructures of EWP/galactomannan mixed gels (category 1) showed that EWP and galactomannans are located in two different phases (Fig. 1; the first and the second column). Since galactomannans have no charge, interactions of EWP and galactomannans are assumed to result in segregative phase separation (Grinberg & Tolstoguzov, 1997). Depletion of galactomannans chains at the surface of the EWP aggregates (Tuinier, Dhont, & de Kruif, 2000) is a probable cause of phase separation. Mixture of negatively charged polysaccharides (categories 2 to 4) and EWP results in more complex structures. Since
some egg white proteins are positively charged at pH 7.0, one would expect both associative and segregative phase separation (Grinberg & Tolstoguzov, 1997) to occur when negatively charged polysaccharides are added. This resulted in more complex microstructures than when only segregative phase separation dictates the microstructure. Moreover, as the pH of the system is closer to the pI of a protein, protein-protein interactions are favored (Ako et al., 2009) which increases the incompatibility between proteins and polysaccharides (de Kruijf & Tuinier, 2001; Picullel & Lindmann, 1992). Thus, addition of polysaccharides further increases protein aggregation and formation of large clusters.

To illustrate this, the effect of polysaccharide concentration on the microstructures of EWP/polysaccharide and WPI/polysaccharide mixed gels at two pH levels are compared (Fig. 9). At pH 7.0, β-lactoglobulin and α-lactalbumin, the two main proteins in WPI, are negatively charged because they are above the pIs of 5.2 and 4.8, respectively (Swaisgood, 1982) and WPI gels have homogenous structures (Çakır & Foegeding, 2011). Combining negatively charged κ-carrageenan with WPI was assumed to result in segregative phase separation (Grinberg & Tolstoguzov, 1997; Çakır & Foegeding, 2011). The structures of the mixed gels were protein continuous, bicontinuous and finally polysaccharide continuous, with sharp phase boundaries as κ-carrageenan concentration increased from 0 to 0.6% (w/w) (Fig. 9; the second row). Depletion interactions increased with increasing κ-carrageenan concentration resulting in a higher degree of phase separation which forces the proteins and polysaccharides in local areas with increased concentration. On the other hand, in the protein micro-phase separated region (pH 5.5), the structure changed from particulate, coarse stranded to polysaccharide continuous as κ-carrageenan concentration increases from 0 to
0.6% (w/w) (Çakır, 2011) (Fig. 9; the third row). At pH 5.5, whey proteins are close to their pI, thus affinity for proteins towards each other as well as positive charge on the proteins increased resulting in both associative and segregative phase separation (de Kruif & Tuinier, 2001) causing a more complex situation and no sharp phase boundaries were observed. Egg white protein/κ-carrageenan mixed gels at pH 7.0 (Fig. 9; the fourth row) were more comparable to WPI/κ-carrageenan mixed gels at pH 5.5 (Fig. 9; the third row) than at pH 7.0 (Fig. 9; the second row). However, the structure of EWP/κ-carrageenan mixed gels did not convert to polysaccharide continuous at a higher κ-carrageenan concentration like WPI/κ-carrageenan mixed gels at pH 5.5. This is possibly due to egg white proteins having a wide range of isoelectric points which helps hold the structures together via associative and segregative phase separation of different proteins.

Moreover, the microstructures of EWP/galactomannan mixed gels (Fig. 9; the first row) were more comparable to WPI/κ-carrageenan mixed gels at pH 7.0 (Fig. 9; the second row). Addition of galactomannans resulted in a microstructure where semi-spherical EWP were embedded in a polysaccharide phase. Galactomannans have no charge density, thus mixed gels assumed to only result in segregative phase separation similar to that of WPI/κ-carrageenan mixed gels at pH 7.0 (Grinberg & Tolstoguzov, 1997). However, phase separated structure of EWP/galactomannan mixed gel did not show a sharp phase boundaries as WPI/κ-carrageenan mixed gels (Fig. 9). The difference could be due to EWP gel structure being micro-phase separated prior to addition of galactomannans, whereas WPI gel was homogenous prior to addition of κ-carrageenan. Further investigation is needed to make a valid conclusion.
Addition of LM pectin resulted in no change in the microstructures of mixed gels (under the micro length scale) (Fig. 1; the fifth column). This trend was also observed by de Jong and van de Velde (2007). Phase separation was prevented due to high osmotic pressure difference between protein and high charge density polysaccharide (de Jong & van de Velde, 2007). However, addition of ι-carrageenan produced structures that were not observed by de Jong and van de Velde (2007). The difference between the microstructures of EWP/LM pectin mixed gel and EWP/ι-carrageenan is possibly due to charge density, molecular weight (MW), and chain stiffness. These factors were shown to be the leading factors determining micro-phase separation (de Jong & van de Velde, 2007).

4.2. Relationship between microstructure, held water and recoverable energy

Egg white protein gels showed some degree of micro-phase separation (Fig. 1). They also had low water holding properties and low elastically stored energy; 38% and 60%, respectively (Fig. 3 to 7). These characteristics of EWP gels fit well with the characteristics of particulate WPI gels (Çakır et al., 2012). Particulate (micro-phase separated) WPI gels showed low RE and HW (42% and 74%, respectively) when compared to RE and HW of homogenous (single phase) WPI gels (60% and 95%, respectively) (Çakır et al., 2012). The relationship between microstructure and ability of gels to hold water in their network had been shown to correlate well (Gwartney, Larick, & Foegeding, 2004; van den Berg et al., 2007a; Çakır & Foegeding, 2011; Çakır et al., 2012; Leksrisompong et al., Submitted). Changes in gel microstructure, especially an increase in porosity, resulted in less water being held during deformation based on Darcy’s equation (van den Berg et al., 2007a) describes flow of fluid through porous material.
Based on energy balance models describing fracture, the total energy supplied to the system (W) can be stored elastically (\(W_e\)), dissipated via the viscous flow (\(W_{d,v}\)), dissipated due to friction process between different components of the gels due to inhomogeneous deformation (\(W_{d,c}\)) or used to cause fracture (\(W_f\)) (van Vliet, Luyten, & Walstra, 1991).

\[
W = W_e + W_{d,v} + W_{d,c} + W_f
\]  \hspace{1cm} (1)

Recoverable energy represents the energy that is elastically stored (\(W_e\)) during deformation and water release represents energy dissipation due to viscous flow (\(W_{d,v}\)) in this equation. Thus, a decrease in RE as the microstructure becomes particulate and coarsens can be explained by high energy dissipation due to water release.

Addition of polysaccharides give rise to different type of microstructures (Fig. 1), which resulted in a wide variety of gelation properties during deformation (Fig. 3 to 7). Similarly, HW and RE of EWP/HM pectin mixed gels decreased as gel porosity increased (Fig. 1; the third column; Fig. 4). However, the ability of the EWP/\(\kappa\)-carrageenan, EWP/LM pectin, and EWP/\(\iota\)-carrageenan mixed gels to store/dissipate energy cannot be explain by water release as explained in the behavior of EWP gels and EWP/HM pectin mixed gels. As the concentration of these polysaccharides increased, there was no changed in RE but the HW increased.

To further investigate this, the effect of polysaccharide type and concentration on breakdown patterns (crack propagation speed within the material) of the mixed gels were investigated. Breakdown pattern is an indirect measurement of the viscoelastic properties of the viscoelastic gels since energy stored and dissipated in the material determines fracture speed (van den Berg et al., 2008). Gels with high elastic components (RE ~ 70 to 85%)
enable them to use the elastically stored energy to fracture via a free-running crack during large deformation resulting in fast fracture (steep breakdown pattern). These gels also had high water holding ability (greater than 90%). On the other hand, gels with high viscous component (RE ~ 30 to 40%) show yielding behavior and as a result, slow fracture propagation. These gels often release large amounts of water during deformation (van den Berg et al., 2008; Çakır et al., 2012). Gel microstructures have also been shown to correlate well with breakdown behavior. The breakdown pattern slopes of homogenous and protein continuous WPI/polysaccharide mixed gels have been shown to have steep breakdown pattern slopes, and the slopes became more gradual as the microstructure of the gels shifted to particulate and coarse stranded (van den Berg et al., 2008; Çakır et al., 2012).

Breakdown patterns of EWP gels (Fig. 3 to 7) were also similar to that of particulate WPI gels (Çakır et al., 2012), which were more gradual than homogenous WPI gels. Addition of 0.1% (w/w) galactomannans caused the slope of the breakdown pattern of mixed gels to become more gradual when compared to EWP gels and RE also became slightly lower (Fig. 3). As for EWP/HM pectin mixed gels, increased HM pectin concentration also resulted in a more gradual slope, which also correlates well with a decrease in RE and HW (Fig. 4). Increasing in concentration of medium and high charge density polysaccharides resulted in no change in the breakdown patterns (Fig. 5C to 7C) which correlates well with no change in RE (elastically stored energy). However, this still does not explain an increased in HW as polysaccharide concentration increased. According to van Vliet et al. (2001)’s equation, these results suggested that the energy could be dissipated via friction ($W_{df}$) or due to micro cracks ($W_j$) that were not observed in RE. Moreover, no effect of polysaccharide
concentration on the microstructure of EWP/LM pectin and EWP/ι-carrageenan mixed gels support no change in breakdown patterns.

4.3. The relationship between microstructure and fracture properties

At pH 7.0, EWP gels showed a mild degree of micro-phase separated structure and had low fracture properties which agree with previous work (Leksrisompong et al., Submitted). Addition of galactomannan concentration above 0.2% (w/w) to the mixed gels caused the discontinuity of the protein network and galactomannan became the continuous phase (Fig. 1) resulting no gelation (Fig. 3). The strength of WPI/galactomanan mixed gels also decreased dramatically when the polysaccharide became the continuous phase (de Jong & van de Velde, 2007). Addition of HM pectin caused the microstructure of the mixed gel to become coarsen and a decreased in fracture stress (Fig.1 and 4A). The fracture surface is reduced as the porosity within the continuous protein network increased resulting in lower gel strength (Walstra, 2003). An increase in gel strength as κ-carrageenan concentration increased (Fig. 5A) was a result of increased local protein concentration in the continuous phase due to micro-phase separation (Fig. 1; the fourth column). This trend also holds true for WPI/κ-carrageenan mixed gels as long as the proteins remain in the continuous phase (Turgeon & Beaulieu, 2001; Tavares & da Silva, 2003; de Jong & van de Velde, 2007; Çakır & Foegeding, 2011). When phase inversion occurs and polysaccharide becomes the continuous phase, the strength of the mixed gels could increase or decrease depending on the concentration and the type (i.e., gelling versus non-gelling) of polysaccharide added to the mixed gels.
However, for high charge density polysaccharides, increased in gel strength cannot be explained by the microstructure since there was no change in the microstructure. Probing the structures at a higher magnification can provide more detailed information about structures of the mixed gels which explains the relationship between the structure and rheological properties (van den Berg, Rosenberg, van Boekel, Rosenberg & van de Velde, 2009). There was also no effect of high charge density polysaccharide concentration on the microstructure of cold set WPI/polysaccharide mixed gel, but an increase in fracture properties was seen as the concentration of \( \iota \)-carrageenan and LM pectin increased (de Jong & van de Velde, 2007). Their explanation for WPI/\( \iota \)-carrageenan mixed gel was that the increased in gel strength was due to syneresis. However, the syneresis was not observed with WPI/LM pectin mixed gel and no explanation was given for that behavior. We also did not observe any syneresis for both EWP/\( \iota \)-carrageenan and EWP/LM pectin mixed gels.

When uniaxial compression is used to determine mechanical properties of soft-solid gels, the correction for water release was shown to produce more accurate results (van den Berg, van Vliet, van der Linden, & van Boekel, 2007b) since gels with higher water release have smaller diameter at a certain strain. However, there was no correction for water release made in this study since fracture properties data from uniaxial compression method showed the same trend and when torsion analysis method was used (Appendix 1). In torsion, deformation occurs in pure shear which minimized shape changing of the samples. Material tested failed according to the weakest point since shear force, compressive and tensile stresses of equal magnitude are created (Diehl, Hamann, & Whitfield, 1979).
4.4 Overall gelation properties of protein/polysaccharide mixed gels

The effect of polysaccharides on microstructure and gelation properties of EWP/polysaccharide mixed gels can be grouped based on the charge density model (de Jong & van de Velde, 2007). It is intriguing how charge density model is valid for grouping the effect of polysaccharide charge density of both acid induced and heat induced of two different protein systems. However, our results showed that polysaccharide charge density model alone cannot be used to predict the ultimate structure and gelation properties of all the mixed gel systems. This is when the effect of individual proteins comes into play and micro-phase separation model (Ako et al., 2009) helps predict the microstructure. The microstructures in turn explain gelation properties (i.e., gel strength and HW) of the mixed gels to a certain degree which agrees with previous finding (van den Berg et al., 2007a and b; de Jong & van de Velde, 2007; Çakır & Foegeding, 2011). However, there are some properties (i.e., RE and breakdown properties) that cannot be explain based on the microstructure at this moment (Fig. 1 and 10).

Fig. 11 is a texture map (a plot of fracture stress versus fracture strain) which describes the textural characteristics of mixed gels (Lanier, 1986). Only a small amount of polysaccharide is required to change the texture of protein gel, especially with addition of κ-carrageenan. Increasing the concentration of κ-carrageenan resulted in a mixed gel with increased strength (fracture stress) and brittle (decreased fracture strain) characteristics (similar to that of WPI gels; Foegeding, 1992). Iota-carrageenan increased gel strength, although less than κ-carrageenan, and had no effect on gel deformability (fracture strain). Both pectins increased fractures strain; making the gels more deformable.
5. **Conclusions**

In a complex system where there are more than just two biopolymers, a combination of polysaccharide charge density model (de Jong & van de Velde, 2007) together with micro-phase separation model (Ako et al., 2009) (i.e., protein charge density) can predict the microstructure of the mixed gels. Based on these microstructures, the gelation properties of EWP/polysaccharide mixed gels can be predicted to a certain extent.

**Acknowledgements**

Support from the North Carolina Agricultural Research Service and American Egg Board are gratefully acknowledged. The use of trade names in this publication does not imply endorsement by the North Carolina Agricultural Research Service of the products named nor criticism of similar ones not mentioned. The authors thank Michael Foods, Danisco, and CP Kelco Inc. for their kind donation of ingredients. The assistance provided by Mrs. JoAnna Foegeding is also acknowledged and greatly appreciated.
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Figure 1. Microstructure of EWP/polysaccharide mixed gels (from left to right column: guar gum, locust bean gum, HM pectin, κ-carrageenan, LM pectin, ι-carrageenan) at four different concentrations (from top to bottom row: 0%, 0.2%, 0.4%, 0.6% (w/w) polysaccharides). Image size: 70.7 x 70.7 μm.
Figure 2. Microstructure of EWP/0.1% (w/w) guar gum (left) and EWP/0.1 (w/w) locust bean gum (right) mixed gels. Image size: 70.7 x 70.7 μm.
Figure 3. Gelation properties of EWP/galactomannan mixed gels as a function of galactomannan concentration; (A) ○ fracture stress, □ fracture strain, (B) Δ RE, ◇ held water, (C) ● 0%, □ 0.1 % (w/w) polysaccharide.
Figure 4. Gelation properties of EWP/HM pectin mixed gels as a function of HM pectin concentration; (A) ○ fracture stress, □ fracture strain, (B) Δ RE, ◇ held water, (C) ● 0%, □ 0.1 %, Δ 0.2%, ◇ 0.3%, Δ 0.4%, ○ 0.5%, ○ 0.6% (w/w) polysaccharide.
Figure 5. Gelation properties of EWP/κ-carrageenan mixed gels as a function of κ-carrageenan concentration; (A) ○ fracture stress, □ fracture strain, (B) Δ RE, ◇ held water, (C) ● 0%, □ 0.1 %, ▽ 0.2%, ◊ 0.3%, Δ 0.4%, ○ 0.5%, ◊ 0.6% (w/w) polysaccharide.
Figure 6. Gelation properties of EWP/LM pectin mixed gels as a function of LM pectin concentration; (A) ○ fracture stress, □ fracture strain, (B) Δ RE, ◇ held water, (C) ● 0%, □ 0.1 %, ▽ 0.2%, ◊ 0.3%, Δ 0.4%, ○ 0.5%, ○ 0.6% (w/w) polysaccharide.
Figure 7. Gelation properties of EWP/t-carrageenan mixed gels as a function of t-carrageenan concentration; (A) ○ fracture stress, □ fracture strain, (B) Δ RE, ◇ held water, (C) ● 0%, □ 0.1 %, ▽ 0.2%, ◊ 0.3%, Δ 0.4%, ○ 0.5%, ○ 0.6% (w/w) polysaccharide.
Figure 8. The effect of polysaccharide type on microstructure of EWP/polysaccharide mixed gels (top) and WPI/polysaccharide mixed gels (bottom; de Jong & van de Velde, 2007). Microstructure images are centered over the polysaccharides.
Figure 9. The effect of polysaccharide concentration on the microstructure of protein/polysaccharide mixed gels (From top to bottom row: EWP/locust bean gum at pH 7.0, WPI/κ-carrageenan at pH 7.0, WPI/κ-carrageenan at pH 5.5, EWP/κ-carrageenan at pH 7.0).
Figure 10. A summary of the effect of polysaccharide type and concentration on gelation properties (● held water; ■ recoverable energy; ◆ fracture stress) of protein/polysaccharide mixed gels.
Figure 11. Texture map describes the textural characteristics of • EWP, Δ guar gum, □ locust bean gum, ◇ HM pectin, ○ κ-carrageenan, ○ LM pectin, ∇ ι-carrageenan.
CHAPTER 5

The Effect of Protein and Polysaccharide Concentration on Freeze/thaw Stability of Egg White Protein Gels

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**Introduction**

A sharp increase in the number of food service and retail stores offering breakfast products in the last years is due to the expanding trends towards eating breakfast on-the-go. These egg products are often precooked, frozen and thawed prior to consumption. Advantages of using frozen pre-cooked egg products are is their convenience for preparation and extended shelf life.

Freezing is a popular and ancient food preservation method because of the relatively high product quality that can be maintained for extended time periods (Person and Londahl 1993). However, there are still some qualities issue associated with frozen products. Freezing is based on the principle of forming ice crystals to separates pure water from solutions (Goff 1992). Quality deterioration of food products often related to ice crystal formation (i.e., crystal size) (Goff 1992), which often causes physical stress and damages the food microstructure (Reid 1990). As results, changes in macroscopic properties such as appearance, water holding properties, flavor, and/or texture after thawing are observed which lower the quality perception by consumers.

Controlling ice-crystal morphology (i.e., size and shape of the ice crystals) had been shown to be one of the most important factors minimizing quality deterioration of frozen and freeze/thaw (F/T) food products (Miyawaki 2001; Aguilera 2005; Rahman 1999; Li and Sun 2001; Goff 2006). Large crystals in ice-cream, which is consumed in frozen state, are perceived to have an icy texture. Smaller ice crystals (< 50 μm) are desired since they cannot be detected by our palate (Hartel 2001). Crystal size and location were also shown to determine the quality of the frozen food that are consumed in the thawed state (i.e., meat
products). Nucleation is the critical step controlling crystal size and distribution (Hartel 2001). Addition of small amounts of hydrocolloids (0.1 to 0.2% w/w) to food products affect the ice crystal size as well as mitigate ice recrystallization and water redistribution. As a result, thaw syneresis is reduced and changes in physical properties induced by F/T is minimized. Typically these ingredients include modified starches and various polysaccharides (Goff 2006).

Despite the growing market opportunities of frozen breakfast products, there are few studies found regarding F/T stability of egg white protein (EWP) gels. One of the first studies investigated F/T stability of EWP gels was done in 1952 by Davis and coworkers. Freezing of cooked EWP gels resulted in rubbery, granular, and high syneresing gels when thawed. These changes were related to physical damage from ice crystals formation (Davis and others 1952). Factors that decrease ice crystal size, such as freezing methods and addition of calcium carbonate (as nuclei), improved water holding properties and increased F/T stability of EWP gels (Davis and others 1952). However, soluble additives (NaCl, glucose, glycerine, tragacanth, karaya, LBG, acacia) showed no improvement on F/T stability since they did not alter ice crystal size (Davis and others 1952; Cotterill 1977). A patent by Hawley and Goves (1970) claimed that addition of 0.5 to 10% water binding carbohydrates (corn, tapioca, rice and potato starches, flours, aligns, carrageenans, agar) aid the F/T stability but no further explanation was given. The most recent study (Gossett and Baker 1983) investigated the effect of pH and succinylation on F/T stability of EWP gels. Altering the protein charge by succinylation and increasing pH were shown to improved F/T stability. Note that these studies were conducted over two decades ago.
Nevertheless, factors affecting the quality of other frozen food products (i.e., ice-cream) have been extensively reviewed and investigated (Goff 1997; 2008; Flores and Goff 1999; Regand and Goff 2003). Hydrocolloids that are often added to ice-cream formation to control crystal size and ice recrystallization include LBG, guar gum, carrageenan and xanthan gum (Regand and Fogg 2003). However, it is hard to apply these findings to investigate the F/T stability of gel based food products since ice-cream is consumed in a frozen state and the ice crystals are what determine the texture of the ice-cream. Freeze/thaw stability of surimi and cooked sausages are more comparable to F/T stability of EWP gels. Addition of ι-carrageenan was shown to improved gel strength and water holding properties of surimi after subjected to two F/T cycles; while addition of κ-carrageenan did not show any improvement (DaPonte and others 1985). Addition of carrageenan controlled ice crystal expansion and their uniformity during F/T. This improved the gelation properties and prevented the spongy, rough texture that is caused by ice crystal growth in the system (DaPonte and others 1985; Bullens and others 1990). Carrageenans were also shown to increase the F/T stability of cooked sausages (DeFreitas and others 1997). This may be related to the physical properties of different carrageenan gels. ι-carrageenan gels are elastic and have good F/T stability. On the other hand, κ-carrageenan gels are known for its firm and brittle characteristics which has poor freeze-thaw stability (Imeson 2000). A blend of κ- and ι-carrageenan increases F/T stability with the assumption that ι-carrageenan imparted the F/T stability to κ-carrageenan gels (Christensen and Trudsoe 1980).

The benefit of using ι-carrageenan to improve F/T stability of gel based food products has been established; however, no clear mechanism(s) has been proposed. Moreover, the
knowledge on how polysaccharides affect F/T stability of cooked EWP gels is still very limited; studies of this topic were conducted over two decades ago and to our knowledge there has not been recent studies published in this area. The objective of this study was to investigate the effect of κ- and ι-carrageenan and protein concentration on F/T stability of EWP gels as determined by water holding properties and microstructures.

**Materials and methods**

**Materials**

Spray dried EWP (82.5% protein, dry basis, N × 6.25) was provided by Michael Foods, Inc. (Minnetonka, MN, U.S.A.). The mineral content was 0.11% P, 0.8% Ca, 0.075% Mg, 0.9% K, 1.4% S and 0.012% Na. Protein and mineral contents were determined by inductively coupled plasma atomic emission spectroscopy. κ-carrageenan (GENUGEL CHP-2F) and ι-carrageenan (GENU TEXTURIZER MB-11F) were kindly provided by CP Kelco Inc. (Lille Skensved, Denmark). Sodium hydroxide (ACS pellets) was purchased from Fisher Scientific Inc. (Fair Lawn, NJ, U.S.A.). Hydrochloric acid was purchased from Mallinckrodt Baker Inc. (Paris, KY, USA). Rhodamine B was purchased from Invitrogen (Eugene, OR, USA). All materials were used without further purification. Deionized water was obtained using a Dracor Water Systems (Durham, NC, U.S.A.) purification system to produce a minimum resistivity of 18.2 MΩ-cm.

**Gel preparation**

**Protein and protein/polysaccharide stock solutions preparation and mixture**

Egg white protein solutions were prepared following the method of Leksrisompong and Foegeding (2011). The pH of EWP 5.0 or 7.0 using 1 N sodium hydroxide or 1 N HCl
prior to final adjustment to 10 or 20% w/v protein and degassed for 1 hr. Polysaccharide solutions (0.2, 0.4% w/w) were prepared by dissolved in deionized water at room temperature (22 ± 2 °C) by stirring overnight.

To prepare protein/polysaccharide stock solutions, the polysaccharide solutions were placed in an 80 °C water bath for 30 min to allow for full hydration. The protein (20% w/v) and polysaccharide stock solutions were then placed in a 45 °C water bath for approximately 15 min. The stock solutions of protein and polysaccharides were then combined at a 1:1 ratio, followed by mechanically stirred.

**Gel formation for held water measurement**

Protein solutions or protein/polysaccharide mixtures were poured into stainless steel tubes (diameter 19 mm) and placed in an 80 °C water bath for 30 min followed by cooling at room temperature (22 ± 1 °C) for 2 hr and refrigeration (4 ± 1°C) overnight.

**Gel formation for microstructure observation**

Gel formation for microstructure observation was performed following the method of Leksrisompong and Foegeding (2011). Rhodamine B (10 μL of a 0.2% (w/w) Rhodamine B + 1 mL of mixtures) was added to protein solution and protein/polysaccharide mixtures prior to pipetted into glass bottom microwell dishes (MatTek corporation, MA, USA) and sealed. The microwell dishes was placed in an 80 °C water bath for 30 min followed by cooling at room temperature (22 ± 1°C) for 2 hr and refrigeration (4 ± 1°C) overnight.

**Freeze/thaw procedure for held water measurement**

The day after preparation, gels were cut into cylinders (10 mm height x 4.3 mm diameter) and placed in the 2.0 mL microcentrifuge filtration tubes. These tubes were placed
in a conventional freezer (Kenmore upright freezer 2826, Hoffman Estates, IL, USA) at -10 ± 1°C for approximately 18 hrs and then removed to the room temperature allowing the gels to thawed for 6 hrs prior to held water measurement. The measurement was done using the microcentrifuge-based method of Kocher and Foegeding (1993). Some of these samples were centrifuged at 159 x g for 10 min using a VWR Symphony 2417R microcentrifuge (VWR International, Radnor, PA, USA). Held water was calculated as the percent of total water remaining in the gel after centrifugation. The samples that were not centrifuged were re-freeze by placed these tubes back in the freezer. The held water was measured at every 0, 1, 3, 5, and 7 days which is corresponding to the number of F/T cycles. Three replications were done for the effect of polysaccharide concentration on F/T stability of EWP/polysaccharide mixed gels. Three to five gel samples were tested per treatment. One replication was done for the effect of protein concentration of F/T stability of EWP gels experiments. Three gel samples were tested per treatment.

**Freeze-thaw procedure for microstructure observation**

Microwell dishes containing gels were placed in a conventional freezer (Kenmore upright freezer 2826, Hoffman Estates, IL, USA) at -10 ± 1°C for approximately 18 hrs and then removed to the room temperature allowing them to thaw for 4 to 6 hrs prior to microstructure observations. Microstructure observations were done using a Zeiss LSM 710 CLSM equipped with an inverted microscope (Zeiss Axio Observer Z1) in the fluorescence mode. The objective lens used was a 40x (LD C-Apochromat 40x/1.1 W Korr M27). The excitation wavelength was at 514 nm and the emission of Rhodamine B was recorded between 546 to 700 nm. The microstructures were observed at 0, 1, 3, 5, and 7 days which is
corresponding to the number of F/T cycles. The same gel samples were used for microstructure observation at all F/T cycles.

Statistically analysis

Data were analyzed using ANOVA using PROC GLM of SAS (version 9.2, SAS Institute Inc., Cary, NC) and comparisons of the means using Tukey’s significant difference test (p < 0.05).

Results and Discussion

Results

Freeze/thaw stability of 10% (w/v) EWP gels prepared at pH 7.0

There was no significant effect of F/T up to 7 cycles on the held water (HW) of 10% (w/v) EWP gels prepared at pH 7.0 (Figure 1A). However, EWP gels showed a high variation in the HW after they were subjected to F/T (standard deviation of HW within the samples tested per treatment ranges from 7 to 15%) (Figure 1A and 2B).

The microstructures of EWP gels are shown in figure 3 and 4. The bright areas represent protein (stained by Rhodamine B) and the dark areas represent the non-protein phase. There was a drastic changed in the microstructures of EWP gels after subjected to the first F/T cycle. Although, increasing F/T cycles showed minimal change in the microstructure (Figure 3 and 4).

Freeze/thaw stability of EWP gels altered by ι-carrageenan

As ι-carrageenan concentration increased, an increase in HW of non-freeze (0 F/T cycle) EWP/ι-carrageenan mixed gels was observed (Figure 1A to C; 0 F/T cycle; lower case letters) which agrees with previous work (Leksrisompong and Foegeding In preparation).
Addition of ι-carrageenan also showed an increasing trend in ability of the mixed gels to hold water after they were subjected to different F/T cycles (Figure 1A to C; lower case letters).

There was no significant effect of F/T cycles on HW of EWP/ι-carrageenan mixed gels at both concentrations (Figure 1B and C). The effect of F/T cycles on F/T stability of EWP/ι-carrageenan mixed gels showed similar trend as F/T stability of EWP gels (Figure 1A to C).

The microstructures of EWP/polysaccharides mixed gels are shown in figure 3 and 4. A degree of micro-phase separated structures were observed when ι-carrageenan was added. The presence of ι-carrageenan coarsens the microstructures of the non-freeze samples (Figure 3 and 4), which agrees with previously observed (Leksrisompong and Foegeding In preparation). Again, there was a drastic changed in the microstructures of EWP/ι-carrageenan mixed gels after the first F/T cycle but increasing F/T cycles resulted in no further change (Figure 3 and 4). Moreover, the microstructures of F/T EWP gel and EWP/ι-carrageenan gels are also similar (Figure 3 and 4).

**Freeze/thaw stability of EWP gels altered by κ-carrageenan**

Increasing κ-carrageenan concentration also increased the HW of non-freeze (0 F/T cycle) EWP/κ-carrageenan mixed gels (Figure 1A, D, and E; lower case letters) which also agrees with previous work (Leksrisompong and Foegeding In preparation). However, when EWP/κ-carrageenan mixed gels (0.1 and 0.2 % w/w) gels were subjected to the F/T, they showed a decreasing trend in HW when compared to F/T EWP gels. For example, EWP/0.1% (w/w) κ-carrageenan mixed gels subjected to 7 F/T cycles had significantly lower HW than EWP gels subjected to 7 F/T cycles (Figure 1A, D and E).
An increased in F/T cycles caused the HW of EWP/0.1% (w/w) κ-carrageenan mixed gels to continued decreasing (Figure 1 D; capital letters). As for EWP/0.2% (w/w) κ-carrageenan mixed gels, HW of mixed gels decreased after the first F/T cycle but increasing F/T cycles did not result in a further decreased in HW of the mixed gels (Figure 1E; capital letters). Again, HW results showed a high standard deviation after the gels were subjected to F/T.

The presence of κ-carrageenan coarsens the microstructures of the non-freeze samples (Figure 3 and 4) which agrees with previously observed (Leksrisompong and Foegeding In preparation). The microstructures of EWP/κ-carrageenan mixed gels changed after subjected to F/T. Again, there was a drastic changed in the microstructures of EWP/κ-carrageenan mixed gels after the first F/T cycle but increasing F/T cycles resulted in no further change (Figure 3 and 4). The pores of F/T EWP/0.1% (w/w) κ-carrageenan mixed gels appear to be larger than those of EWP/0.2% (w/w) κ-carrageenan mixed gels (Figure 3 and 4). Moreover, pores of F/T EWP/κ-carrageenan mixed gels were also much larger than F/T EWP gels and EWP/ι-carrageenan mixed gels (Figure 3 and 4).

Freeze/thaw stability of EWP gels altered by protein concentration

Increasing protein concentration increased the HW of non-freeze EWP gels (0 F/T cycle) prepared at pH 7.0 and 5.0 (Figure 2 and 5; lower case letters). This was expected since increased in protein concentration is known to increase HW of the globular protein gels (Verhuel and Roefs 1998). Moreover, EWP gels at pH 7.0 showed higher HW than prepared at pH 5.0 at all protein concentration (Figure 2 and 5) which agrees with previous researchers
When the samples were subjected to F/T, high standard deviation in HW of F/T EWP gels and increasing F/T cycles increased HW in some treatments. However, smaller standard deviation were observed within the treatments at higher protein concentration and higher protein concentration showed a significant increased in HW after the gels were subjected to F/T (Figure 2 and 5; lower case letters).

The structural changed was observed after the protein gels were subjected to F/T but minimal microstructural change was seen at higher protein concentration (Figure 6 to 9). At higher protein concentration, smaller pores were also observed for EWP prepared at both pH conditions.

**Discussion**

Out of all polysaccharide treatments observed in this study, addition of 0.2% ι-carrageenan to EWP gels resulted in the highest F/T stability as determined by HW (Figure 1; lower case letters) and microstructure (Figure 3 and 4). κ-carrageenan gels are known to be firm and brittle with poor F/T stability, whereas ι-carrageenan gels are elastic and have good F/T stability (Imeson 2000). As for protein concentration effects on EWP F/T stability, the data showed that higher protein concentration increase F/T stability of protein gels prepared at pH 5.0 and 7.0 as determined by HW and microstructure. The relationship between microstructure and HW of gels has been shown to be highly correlated; decreasing in gel porosity resulted in higher ability for gels to hold water when subjected to deformation (Gwartney and others 2004; van den Berg and others 2007; Cakir and Foegeding 2011;
Leksrisompong and others Submitted). However, this relationship was not observed in our study.

However, it is hard to draw a valid conclusion since the HW results show high standard deviation. In addition to high standard deviation, increasing F/T cycles resulted in an increased in HW for some treatments (Figure 2 and 5). Moreover, visual observations of the F/T EWP gels and EWP/polysaccharide mixed gels for all the treatments showed that there was great variation between samples of one treatment. For instance, two out of five gel samples tested per treatment showed macroscopic defects at visual length scale whereas the other three samples remained unchanged. Macroscopic defect samples resulted in a lower HW than normal samples which causes a high fluctuation in HW within the treatments. These are indications of problems associated with

1. Method used to measure water holding properties (the free water is not being released due to particles plugging the filter)
2. Freeze/thaw procedure.

**Future work**

Ice crystals is one of the most important factors determining the quality of F/T products (Miyawaki 2001; Aguilera 2005; Rahman 1999; Goff 2006). Three phenomena were proposed to be involved in ice crystallization of water (Figure 10) (Hartel 2001): 1. Nucleation: formation of crystalline structure from the solution; 2. Ice crystal growth, a subsequent growth until the equilibrium is attained and 3. Recrystallization: ice crystals undergo reorganization of crystalline structure as well as changing in size, shape, and number during storage to lower energy state. Thus, any parameters affecting these phenomena need to be controlled when designing an experiment. The examples of these parameters include
freezing rate and storage temperature. Freezing rate determines the nucleation formation. Rapid freezing enhances ice nucleation formation resulting in a large number of ice crystals produced but smaller in size. Inconsistent freezing rate results in different ice crystal size. Constant and subzero storage temperature with minimal temperature fluctuations are important for quality in frozen foods (Reid 1990). Fluctuation leads to the process of ice recrystallization which develops fewer ice crystals but larger in size. Packaging of the frozen sample is also important since it protects samples from freezer burn due to sublimation and dehydration. These factors make F/T stability a challenging subject to study.

Moreover, protein/polysaccharide interactions should also be considered when investigating the effect of polysaccharide on F/T stability of food products. Gelation properties of protein/polysaccharide mixed gels were shown to depend on their molecular properties and solvent conditions (de Jong and van de Velde 2007; Çakır and Foegeding 2011; Leksrisompong and Foegeding In preparation). Mixture of biopolymers often lead to associative (interactions between biopolymers) or segregative (no interactions between biopolymers) phase separation which in turn affect the properties of biopolymer mixtures and potentially will affect their F/T stability. Furthermore, it needs to determined whether observing the structures at the micro-length scale is sufficient to understand the F/T stability of the mixed gels. It was shown that a combination of probing the structures at micro and submicron length scale provide detail information regarding the structure/property relationship of the mixed gel system since biopolymer interactions and spatial distribution cannot be observed under the micro-length scale (van den Berg and others 2009). Lastly, the
future study needs to verify if held water is an appropriate method used to measure water holding properties of F/T samples.
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Figure 1. Freeze thaw stability of A. EWP gels; B. EWP/0.1% (w/w) \( \iota \)-carrageenan; C. EWP/0.1% (w/w) \( \iota \)-carrageenan mixed gels; D. EWP/0.1% (w/w) \( \kappa \)-carrageenan; E. EWP/0.2% (w/w) \( \kappa \)-carrageenan prepared at pH 7.0. Lower case letters indicate significant difference between freeze/thaw cycles of gels prepared with different polysaccharide type and concentration. Lower case italic letters indicate significant difference between freeze/thaw cycles of gels prepared with \( \kappa \)-carrageenan. Capital letters indicate significant difference within each polysaccharide treatment.
Figure 2. Freeze/thaw stability of EWP gels prepared at pH 7.0 and various protein concentrations: A. 8% (w/v); B. 10% (w/v); C. 12% (w/v); D. 14% (w/v); E. 16% (w/v); F. 18% (w/v); G. 20% (w/v). Lower case letters indicate significant difference between freeze/thaw cycles of gels prepared with different protein concentration. Capital letters indicate significant difference of gels within each protein concentration.
Figure 3. CLSM images of EWP gels and EWP/polysaccharide mixed gels prepared at pH 7.0 after subjected to: A. 0 cycle; B. 1 cycles; C. 3 cycles; D. 5 cycles; E. 7 cycles of freezing and thawing. Image size: 70 x 70 μm.
Figure 4. CLSM images of EWP gels and EWP/polysaccharide mixed gels prepared at pH 7.0 after subjected to: A. 0 cycle; B. 1 cycles; C. 3 cycles; D. 5 cycles; E. 7 cycles of freezing and thawing. Image size: 425 x 425 μm.
Figure 5. Freeze thaw stability of EWP gels prepared at pH 5.0 and various protein concentration A. 8% (w/v); B. 10% (w/v); C. 12% (w/v); D. 14% (w/v); E. 16% (w/v); F. 18% (w/v); G. 20% (w/v). Lower case letters indicate significant difference between freeze/thaw cycles of gels prepared with different protein concentration. Capital letters indicate significant difference of gels within each protein concentration.
Figure 6. CLSM images of 8, 10, 12, 14, 16, 18, 20% (w/v) EWP gels prepared at pH 7.0 after subjected to: A. 0; B. 1; C. 3; D. 5; E. 7 cycles of freezing and thawing. Image size: 70 x 70 μm.
Figure 7. CLSM images of 8, 10, 12, 14, 16, 18, 20% (w/v) EWP gels prepared at pH 7.0 after subjected to: A. 0; B. 1; C. 3; D. 5; E. 7 cycles of freezing and thawing. Image size: 425 x 425 μm.
Figure 8. CLSM images of 8, 10, 12, 14, 16, 18, 20% (w/v) EWP gels prepared at pH 5.0 after subjected to: A. 0; B. 1; C. 3; D. 5; E. 7 cycles of freezing and thawing. Image size: 70 x 70 μm.
Figure 9. CLSM images of 8, 10, 12, 14, 16, 18, 20% (w/v) EWP gels prepared at pH 6.0 after subjected to: A. 0; B. 1; C. 3; D. 5; E. 7 cycles of freezing and thawing. Image size: 425 x 425 μm.
Figure 10. Schematic illustrating phenomena involved in crystallization of water (Hartel 2001).
APPENDICES
APPENDIX 1

Comparison Between Fracture Properties Data of EWP/Polysaccharide Mixed Gels from Torsion Analysis versus Uniaxial Compression Methods

(Data From Chapter 4)
Figure 1. Gelation properties of EWP/galactomannan mixed gels as a function of galactomannan concentration ○ uniaxial compression; □ torsion analysis.
Figure 2. Gelation properties of EWP/HM pectin mixed gels as a function of HM pectin concentration ○ uniaxial compression; □ torsion analysis.
Figure 3. Gelation properties of EWP/κ-carrageenan mixed gels as a function of κ-carrageenan concentration ○ uniaxial compression; □ torsion analysis.
Figure 4. Gelation properties of EWP/LM pectin mixed gels as a function of LM pectin concentration ○ uniaxial compression; □ torsion analysis.
Figure 5. Gelation properties of EWP/ι-carrageenan mixed gels as a function of ι-carrageenan concentration ○ uniaxial compression; □ torsion analysis.
APPENDIX 2

Effect of flavor on perceived texture of whey protein isolate gels

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EFFECT OF FLAVOR ON PERCEIVED TEXTURE OF WHEY PROTEIN ISOLATE GELS

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ABSTRACT

This study investigated the role of flavor on trained panelist and consumer perception of texture properties. Whey protein isolate (WPI) gels were prepared with sodium chloride (25 mM) at different pH (pH 6.0 or 7.0), and calcium chloride concentrations (0 or 10 mM). The same gel treatments were produced with and without added flavor. Instrumental torsion analysis showed that flavor addition had no impact (P > 0.05) on fracture stress and strain. Texture properties of gels were evaluated by a trained descriptive panel and untrained consumers (n = 60) for their perception of gel firmness, fracturability, juiciness, mouth coating and overall disliking. Distinct texture properties among the gels were documented by both trained panelists and consumers (P < 0.05). Flavor addition did not influence (P > 0.05) texture perception. Therefore, flavor addition does not impact panelist (trained or untrained) ability to evaluate textural differences in WPI gels.

PRACTICAL APPLICATIONS

Much current work is focused on understanding oral processing and the sensory perception of texture. Many studies have evaluated the influence of texture on flavor perception, and many of those that have looked at the effect of flavor on texture perception have not controlled for rheological differences in texture. As such, the influence of flavor on texture perception by trained panelists and/or untrained consumers is not well documented. A better understanding of these effects would (1) help researchers studying mastication

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behaviors and/or food texture to create more palatable, flavorful samples without compromising the textural integrity of their samples, and (2) help commercial food manufacturers more easily understand drivers of consumer liking when pursuing new product development or line extensions.

INTRODUCTION

Flavor and texture perception in foods are complex phenomena because of interactions between taste, aroma and texture modalities (González-Tomás et al. 2008; Tournier et al. 2009). Much research has been conducted on these interactions, but most has focused on the influence of texture on flavor perception. The influence of texture on flavor intensity and sweetness has been well documented (Pangborn et al. 1973; Pangborn and Szczesniak 1974; Pangborn et al. 1978; Guinard and Marty 1995; Theunissen and Kroze 1995; Weel et al. 2002; Lethuaut et al. 2003; Bayarri et al. 2006). For example, studies have shown that flavor release and flavor intensity are influenced by gel texture (Guinard and Marty 1995; Weel et al. 2002). In contrast, using a panel familiar with assessing dairy products, González-Tomás et al. (2008) reported that viscosity and orally perceived thickness did not influence strawberry flavor intensity perception in dairy desserts.

Some studies have evaluated the effect of presence or concentration of flavor or basic taste on texture perception. Burns and Noble (1985) showed that when viscosity was controlled in vermouth, samples with higher sucrose levels were perceived to be more viscous by a trained panel. Jaime et al. (1993) showed that flavor type and flavor level (0.75% versus 1.25% raspberry flavor and 0.5% versus 1.0% butterscotch flavor) in gelatin desserts did not significantly influence texture perception by a trained panel. However, in their study, presence or absence of flavor was not evaluated (Jaime et al. 1993). More recently, Tournier et al. (2009) suggested that the consumer texture perception of custard desserts with the same rheological properties was not altered by taste and aroma. However, much of the previous research that has focused on the effect of flavor, aroma, and/or basic tastes on sensory texture has discounted the effect of instrumental texture differences. Lethuaut et al. (2003) found that sucrose level affected texture perception by trained panelists in model dairy desserts. However, instrumental texture differences also existed between samples. Similarly, Stampanoni and Noble (1991) showed that increasing salt and acid increased trained panelist perception of cheese analogs, although rheological results showed texture differences as well.

Previous research conducted on whey protein isolate (WPI) gels has shown that pH and different salt concentrations and types can alter the microstructure and rheological properties of the gels (Gwartney et al. 2000, 2004).
WPI is used in a wide variety of food applications and WPI gels with distinct texture and rheological properties can be readily produced (Gwartney et al. 2004; Barrangou et al. 2006). Many studies have only evaluated the influence of texture on flavor perception, and many of those that looked at the effect of flavor on texture perception did not control for rheological differences in texture. Because the influence of flavor and taste on texture perception is not well understood (Lethuaut et al. 2003), the objective of this study was to determine if the presence of flavor influenced sensory texture perception in various types of WPI gels. Instrumental analysis, a trained descriptive panel, and consumer testing were applied to achieve this objective.

MATERIALS AND METHODS

Materials

A commercial WPI (94.21% protein, dry basis, based on measurement of nitrogen by inductively coupled plasma spectroscopy) was supplied by Davisco Foods International, Inc. (Le Sueur, MN). Sodium hydroxide (ACS pellets) was purchased from Fisher Scientific Inc. (Fair Lawn, NJ). Sodium chloride (NaCl) and calcium chloride (CaCl₂) were purchased from Sigma-Aldrich (St. Louis, MO). Vanilla flavor, containing both natural and artificial flavors (#457322), was provided by Flavor Systems International, Inc. (Cincinnati, OH); strawberry flavor, containing both natural and artificial flavors (#149451), was provided by Mother Murphy’s Laboratories, Inc. (Greensboro, NC). Deionized water (>17 MΩ) was obtained using a Dracor Water Systems (Durham, NC) purification system.

METHODS

Preparation of Protein Solutions and Gelation

WPI was mixed with deionized water and stirred at room temperature (22 ± 2°C) to allow full hydration. NaCl (25 mM) was added to all WPI solutions prior to pH adjustment and when required, 10 mM CaCl₂ was also added. Each treatment was prepared with no flavor, strawberry and vanilla flavor; flavors were added at a final concentration of 0.2% (w/v). The pH of the WPI solution was then adjusted to 6.0 or 7.0 using 1 N NaOH or 1 N HCl prior to final adjustment to 12% w/v protein and degassed for 1 h. WPI solutions were filled into glass tubes (inner diameter 19 mm) and closed by a rubber stopper at the bottom (Barrangou et al. 2006). Tubes were covered with aluminum foil to prevent evaporation. The solutions were heated at 80°C in a water
bath for 30 min, followed by cooling at room temperature (22 ± 2°C) for 1 h and overnight refrigeration at 4°C. Gels were allowed to equilibrate at room temperature (22 ± 2°C) for at least 1 h before rheological or sensory analysis. Each gel treatment was prepared in duplicate.

**Total Moisture Content**

The total moisture content of gels was measured according to AOAC (1995) methodology. Approximately 3 g of sample was weighed into aluminum pans and dried in a 100°C oven for 12 h. Total moisture content (% w/w) of each gel was measured over three replications and results were further used in the calculation of water-holding capacity (WHC).

**WHC**

The WHC of each gel type was measured using the microcentrifuge-based method of Kocher and Foegeding (1993). Each treatment was performed in triplicate. A cork borer was used to cut the gels into 10 x 4.8 mm cylinders. Cut gels were then inserted into the microcentrifuge filtration unit which consisted of a 2.0-mL microcentrifuge tube (Beckman Instruments, Inc., Palo Alto, CA) that collected released fluid and an inner tube that held the sample. Samples were centrifuged at 153 x g (2,000 rpm) for 10 min.

**Rheological Analysis**

Torsion analysis was used for the measurement of large strain rheology, namely fracture stress and strain, as described by Kim *et al.* (1986). Gels were cut into 28.7 mm long pieces. Notched plastic discs approximately 27 mm in diameter and 1 mm thick (Gel Consultants, Inc., Raleigh, NC) were glued to each end of the cylindrical gel piece using instant adhesive cyanoacrylate glue ("Quickite" Super Glue, Loctite Corp., Cleveland, OH). The cylinders were mounted on a modified milling machine and ground into a dumbbell-shape with a center diameter of 10 mm, then mounted on the Hamann Torsion Gelometer (Model GC-TG92 US, Gel Consultants, Inc.) to measure fracture stress and fracture strain of the gels. Samples were twisted to fracture at 2.5 rpm (corresponding to a strain rate of 0.26/s) (Barrangou *et al.* 2005). Fracture stress (strength) and fracture strain (deformation) were calculated with the Torsion Gelometer software (Gel Consultants Inc.).

**Descriptive Sensory Analysis**

All sensory testing was conducted in accordance with the North Carolina State University Institutional Review Board for Human Subjects guidelines. Evaluation of WPI gel texture was conducted using a trained descriptive
sensory panel and an established cheese texture lexicon (Brown et al. 2003) modified for WPI gel texture analysis. Eight panelists (all female; ages 30–56 years) performed descriptive analysis of gel texture using the Spectrum™ method (Meilgaard et al. 1999; Drake and Civille 2003). Each panelist had more than 1,000 h of descriptive analysis experience on various products, with 250 h of texture-specific experience. Panelists spent 4 h familiarizing themselves with the WPI gels, and reviewing and discussing texture terms as they pertained to WPI gels.

For sensory analysis, gels were cut at room temperature (22 ± 2°C) 2 h prior to analysis to prevent any gel deformation or water loss. Gels were cut into cylinders (15 mm in height, 19 mm in diameter) and dispensed (7 cylinders per treatment per cup) into lidded 112-mL soufflé cups with random three-digit codes. Each panelist independently evaluated the texture attributes of each gel using a 0–15-point product-specific scale (Brown et al. 2003). The evaluation of gel texture attributes included both hand and mouth terms. Three texture terms were used to describe texture characteristics perceived by hand: hand firmness, hand springiness and rate of recovery. Mouth evaluation terms were broken into two parts: first bite (firmness and fracturability) and chew down (degree of break down, cohesiveness, adhesiveness, moisture release, smoothness and residual mouth coating). These attributes were evaluated using the same lexicon described by Brown et al. (2003) with one additional term – moisture release, which was defined as the degree of moisture release from the sample while chewing. Reference samples were provided for each attribute at each assessment. Panelists were instructed to expectorate gels, and deionized water was provided for palate cleansing. Over the course of two separate sessions, each panelist evaluated every sample in duplicate using a randomized balanced design. Compusense® five version 4.6 (Compusense, Guelph, Canada) was used for data collection.

Consumer Testing

Based on the descriptive analysis results, three types of WPI gels (pH 6.0 with CaCl₂, pH 7.0 with CaCl₂, and pH 7.0 without CaCl₂) with and without one flavor (vanilla) were selected for consumer testing (6 samples total). These gels were selected to represent a wide range of texture properties with and without flavoring. Consumers (n = 60, individuals with no descriptive analysis experience) with no known allergies to milk or milk products were recruited and included students, faculty and staff at North Carolina State University (Raleigh, NC). Each gel was cut into cylinders as for descriptive analysis and dispensed into lidded 112-mL soufflé cups with three-digit codes. Gels were served using a completely randomized design. Because the appearance of different gels was quite distinct and visual cues might influence untrained
consumers, precautions were taken to ensure that panelists could not see the samples before or during consumption. Specifically, the samples were kept in black, opaque, lidded containers; the panel was conducted in a darkened room; and panelists were blindfolded during sample assessment to prevent any bias that might be caused by sample appearance. A researcher recorded each consumer’s response on a paper ballot.

Two pieces from each gel were given to assess the textural properties. The first piece was used to evaluate the firmness and fracturability of the gel by one complete bite, using the molars, through the sample. Panelists assessed the moisture release and residual mouth coating using the second piece. Each of these properties was assessed using a 9-point intensity scale where 1 = low intensity and 9 = very high intensity. Finally, panelists indicated their overall disliking for the sample. The disliking scale was a unipolar 9-point scale, where 1 = no opinion and 9 = the worst product you have ever consumed (Herr and Page 2004). This scale was selected over the 9-point hedonic scale because gels were not expected to be liked by consumers and it was desired to maximize the potential to detect differences in degree of perceived dislike (Herr and Pages 2004). Panelists were asked to expectorate the gels after evaluation and to rinse their palates with deionized water between samples.

Statistical Analyses

Rheological, descriptive sensory analysis and quantitative consumer data were first analyzed independently, and then the latter two were also analyzed together. A two-way analysis of variance (General Linear Model with Tukey’s Honestly Significant Difference test as a post hoc test, SAS, version 9.1, Cary, NC [SAS 1999]) was applied. Principal component analysis (XLSTAT statistical software version 2006.3, Addinsoft, New York, NY) was also used to analyze the descriptive data. Partial least squares (PLS) regression analysis was used to analyze the combination of descriptive and quantitative consumer data.

RESULTS AND DISCUSSION

WHC

WPI gels prepared at pH 7.0 without CaCl₂ showed the highest WHC, followed by the gels prepared at pH 7.0 with 10 mM CaCl₂, and both gels prepared at pH 6.0 (Fig. 1). These results are consistent with previous studies in which particulate WPI gels (gels formed under conditions where there is minimal charge repulsion) held less water than stranded WPI gels (gels formed under conditions where there is high charge repulsion) (Langton and Hermans-
FIG. 1. WATER-HOLDING CAPACITY OF WHEY PROTEIN ISOLATE GELS (12% w/v) PREPARED AT pH 6.0 AND pH 7.0, WITH 0 AND 10 mM CaCl$_2$
Treatments with different letters were found to be significantly different.

son 1992; Gwartney et al. 2004). In this study, gels prepared at pH 7.0 without CaCl$_2$ behaved like fine-stranded gels while all other gels behaved like particulate gels. Gels prepared at pH 7.0 with CaCl$_2$ formed a particulate gel structure because of charge screening and aggregation due to higher ionic strength. When the pH was decreased to 6.0, the pH of the system was shifted toward the isoelectric point of WPIs, which lowered the charge repulsion and caused particulate gel network formation.

Instrumental Analysis

Particulate gels prepared at pH 7.0 with the addition of 10 mM CaCl$_2$ had higher fracture stress values compared with stranded gels made at pH 7.0 without CaCl$_2$. Decreasing the pH to 6.0 produced softer gels as indicated by lower fracture stress values (Fig. 2). However, there was no significant difference ($P > 0.05$) in fracture stress between gels with flavor or without flavor.

Stranded gels prepared at pH 7.0 without CaCl$_2$ showed the highest deformability while particulate gels at pH 7.0 with 10 mM CaCl$_2$ were lower (Fig. 3). These results are in agreement with Gwartney et al. (2004) who found a similar difference between particulate and stranded WPI gels. The effect of CaCl$_2$ varied depending on the pH. At pH 6.0, the addition of CaCl$_2$ increased the fracture strain, but at pH 7.0, a significant decrease was observed with the addition of CaCl$_2$. Once again, there was no significant difference ($P > 0.05$) in deformability within a treatment by the addition of flavoring compounds.
Descriptive Sensory Analysis

Descriptive analysis is an analytical tool where trained panelists (usually 6–12) perform analogously to an instrument to identify and quantify attributes of a product (Drake 2007), in this study, gel texture. Another purpose of conducting descriptive analysis in this study was to narrow down the number of samples for consumer testing. Differences were documented between different gel types (Table 1). These results are consistent with findings by Gwartney et al. (2000) who demonstrated that panelists could detect differences between WPI gel textures. The texture of stranded WPI gels (pH 7.0 without CaCl₂) was clearly different than the particulate WPI gels (pH 6 without CaCl₂ and pH 7.0 with CaCl₂). WPI gels prepared at pH 6.0 with addition of CaCl₂ were also particulate but these gels were distinct in texture from other treatments. For instance, these gels (treatments 4, 5 and 6 in Table 1) had greater breakdown, moisture release, adhesiveness, residual mouth coating and firmness than the remaining gels. The rest of the gels were smoother, higher in hand springiness, had higher fracturability and had higher rates of recovery. Solvent conditions, salt in this case, affect the gel microstructure because they
FIG. 3. FRACTURE STRAIN OF 12% (W/V) WHEY PROTEIN GELS PREPARED AT pH 6.0 AND pH 7.0 WITH 0 AND 10 mM CaCl₂

Each treatment was prepared with no flavor, vanilla flavor (0.2% w/v) and strawberry flavor (0.2% w/v). Results are the mean of at least two replicates, and error bars represent ± standard deviation. Treatments with different letters were found to be significantly different.

influence the way proteins associate to form a gel network. In turn, the microstructure has a great impact on WHC, gel texture and gelation properties (fracture stress and fracture strain).

As expected by a trained panel, addition of flavor, vanilla or strawberry, resulted in no differences in any gel texture attribute perceived by panelists within the same gel type (Table 1). Conceptually, trained panelists are similar to components of an instrument (Drake 2007). Flavor or visual differences ideally should have no impact on perception of texture properties by trained panelists unless flavor has a cross-modal impact on texture perception, which was not expected. However, some studies have demonstrated that texture can influence the trained panelist’s perception of flavor. Gwartney et al. (2000) showed that trained panelists perceived higher maximum intensity of flavor release and release rate of gels with particulate structures. Both Jaime et al. (1993) and Guinard and Marty (1995) conducted studies on gelatin gels and their results showed that as the gel hardness increased, the maximum flavor intensity perceived and overall gel flavor diminished. On the other hand, Lethuaut et al. (2003) looked at the effect of sucrose concentration and carrageenan type on texture perception and found that increased sucrose concent-
### Table 1

**EFFECT OF FLAVOR ON TEXTURE FOR WHEY PROTEIN ISOLATE GELS BY DESCRIPTIVE SENSORY ANALYSIS**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Hand evaluation</th>
<th>Mouth evaluation – First bite</th>
<th>Mouth evaluation – Chew down</th>
<th>Mouth evaluation – Residual</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Firmness</td>
<td>Springiness</td>
<td>Rate of recovery</td>
<td>Firmness</td>
</tr>
<tr>
<td>pH6 NF</td>
<td>AB 11.3</td>
<td>AB 14.8</td>
<td>AB 15.0</td>
<td>AB 3.21</td>
</tr>
<tr>
<td>pH6 Vanilla</td>
<td>AB 11.3</td>
<td>AB 15.0</td>
<td>AB 14.8</td>
<td>AB 3.17</td>
</tr>
<tr>
<td>pH6 Strawberry</td>
<td>AB 5.6</td>
<td>B 14.8</td>
<td>A 15.0</td>
<td>A 2.77</td>
</tr>
<tr>
<td>pH6 NF CaCl₂</td>
<td>AB 5.79</td>
<td>C 13.3</td>
<td>A 13.5</td>
<td>B 6.03</td>
</tr>
<tr>
<td>pH6 Vanilla CaCl₂</td>
<td>AB 5.59</td>
<td>C 14.5</td>
<td>A 14.5</td>
<td>AB 6.79</td>
</tr>
<tr>
<td>pH6 Strawberry CaCl₂</td>
<td>AB 6.44</td>
<td>C 14.3</td>
<td>A 14.4</td>
<td>AB 6.63</td>
</tr>
<tr>
<td>pH7 NF</td>
<td>AB 12.9</td>
<td>A 15.0</td>
<td>A 15.0</td>
<td>A 3.64</td>
</tr>
<tr>
<td>pH7 Vanilla</td>
<td>AB 12.7</td>
<td>AB 15.0</td>
<td>A 15.0</td>
<td>AB 5.20</td>
</tr>
<tr>
<td>pH7 Strawberry</td>
<td>AB 13.9</td>
<td>A 15.0</td>
<td>A 15.0</td>
<td>AB 3.49</td>
</tr>
<tr>
<td>pH7 NF CaCl₂</td>
<td>AB 11.6</td>
<td>AB 15.0</td>
<td>A 15.0</td>
<td>AB 4.14</td>
</tr>
<tr>
<td>pH7 Vanilla CaCl₂</td>
<td>AB 12.5</td>
<td>AB 14.8</td>
<td>A 14.9</td>
<td>AB 3.02</td>
</tr>
<tr>
<td>pH7 Strawberry CaCl₂</td>
<td>AB 12.2</td>
<td>AB 15.0</td>
<td>A 15.0</td>
<td>AB 3.30</td>
</tr>
</tbody>
</table>

Attributes were scored on a 0-15-point product-specific scale (Brown et al., 2003); values represent the mean scores assigned by trained panelists. A-C indicates that means within columns with different letters are statistically different (P< 0.05).

NF, no flavor.
tration changed perceived texture attributes; however, these perceptions could be because of changes in mechanical profile as there were also changes in the rheological data. Jaime et al. (1993) found that gelatin concentration and flavor type changed the perception of flavor, but type and level of flavor did not have much effect on texture perception, corresponding with the current findings.

**Consumer Perception**

Based on the descriptive analysis results (Fig. 4), three distinct gel textures were selected for consumer testing. Flavor did not influence the trained panelists’ evaluation of texture, as expected, but it was unknown whether or not consumers could differentiate flavor differences from textural differences. Results indicated that flavor did not significantly \( P < 0.001 \) impact consumer perception of gel texture attributes. Specifically, gel treatments that differed only by flavor addition were perceived by consumers as having the same firmness, fracturability, moisture release and residual mouth coating properties (Table 2). Furthermore, the degree of disliking did not vary with flavor addition, indicating that the addition of vanilla flavor did not

![Biplot (axes F1 and F2: 93%)](image)

**FIG. 4. PRINCIPAL COMPONENT (PC1 AND PC2) BI PLOT OF DESCRIPTIVE ANALYSIS OF WHEY PROTEIN ISOLATE (WPI) GEL TEXTURE ATTRIBUTES**

WPI gel treatments are represented by numbers (Table 1).
TABLE 2.
EFFECT OF FLAVOR ON CONSUMER PERCEPTION OF TEXTURE FOR WHEY PROTEIN ISOLATE GELS

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Firmness</th>
<th>Fracturability</th>
<th>Moisture release</th>
<th>Mouth coating</th>
<th>Degree of dislike</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 6 + CaCl₂</td>
<td>3.0 ± 1.7a</td>
<td>3.3 ± 1.94a</td>
<td>6.5 ± 1.6a</td>
<td>5.6 ± 2.2a</td>
<td>6.8 ± 2.0a</td>
</tr>
<tr>
<td>pH 6 + CaCl₂ + flavor</td>
<td>3.5 ± 1.6a</td>
<td>3.6 ± 1.98a</td>
<td>6.3 ± 1.8a</td>
<td>5.8 ± 2.0a</td>
<td>6.6 ± 2.0a</td>
</tr>
<tr>
<td>pH 7</td>
<td>4.6 ± 2.0a</td>
<td>4.4 ± 2.31a</td>
<td>2.3 ± 1.4a</td>
<td>2.0 ± 1.2a</td>
<td>3.9 ± 1.9a</td>
</tr>
<tr>
<td>pH 7 + flavor</td>
<td>5.0 ± 1.9a</td>
<td>4.2 ± 1.93a</td>
<td>2.4 ± 1.5a</td>
<td>2.4 ± 1.5a</td>
<td>3.9 ± 2.0a</td>
</tr>
<tr>
<td>pH 7 + CaCl₂</td>
<td>5.2 ± 2.0a</td>
<td>6.5 ± 1.67ca</td>
<td>1.9 ± 1.2ca</td>
<td>4.0 ± 2.1ca</td>
<td>5.3 ± 2.1ca</td>
</tr>
<tr>
<td>pH 7 + CaCl₂ + flavor</td>
<td>5.1 ± 1.9a</td>
<td>6.1 ± 1.74ca</td>
<td>2.0 ± 1.1ca</td>
<td>3.7 ± 2.0ca</td>
<td>4.9 ± 2.0ca</td>
</tr>
<tr>
<td>Gel × flavor interactions</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

Arithmetic mean ± standard deviation of consumer responses using a 9-point intensity or hedonic dislike scale where 1 = low intensity or no opinion and 9 = high intensity or dislike extremely, respectively. Uppercase letters within a single column differentiate gel texture treatments that were distinguished by consumers as being significantly different (P < 0.001). Lowercase letters within a single column differentiate flavor treatments that were distinguished by the same consumers as being significantly different (P < 0.05). There were no significant interactions between flavor and gel texture.

improve palatability. These results suggest that the unusual textural properties of each gel were the main cause of dislike. Overall, panelists most disliked the gel texture obtained at pH 6 with CaCl₂. This gel was rated highest for moisture release and mouth coating and low in firmness and fracturability, suggesting that consumers may not like those texture attributes.

Independent of flavor addition, consumers were able to differentiate texture properties between treatments (Table 2). However, the addition of CaCl₂ to the gels at pH 7.0 did not impact consumer perception of firmness, even though the instrumental data showed significant differences in fracture stress (Fig. 2) or strain (Fig. 3). Likewise, trained panelists also did not detect differences in hand firmness or first-bite mouth firmness between the pH 7.0 treatments with and without CaCl₂ (Table 2). The lack of agreement between sensory and instrumental perception for those specific gel texture attributes could be a result of differences between instrumental and sensory sensitivity (Foegeding and Drake 2007). There were no significant interactions between flavor and gel texture for any of the attributes.

Multivariate statistical analyses were conducted to determine the relationship between the consumer and descriptive data. The two-factor PLS plot in Fig. 5 shows all attributes as determined by both consumer and trained panelists. The plot demonstrates that consumers and trained panelists were able to distinguish between different gel treatments and that the addition of flavor did not change the way they perceived the gels. Both consumers and trained panelists had a similar understanding of the fracturability, moisture
release and residual mouth coating, but consumers interpreted gel firmness differently from the trained panel (Fig. 5). Both consumers and trained panelists evaluated firmness via the first-bite approach; trained panelists alone evaluated firmness by the additional hand-compression method. The difference in firmness perception is likely because of the training each panelist received. For instance, trained panelists receive many hours of training using a 0–15-point scale anchored with verbal descriptions and physical samples. In contrast, consumers received no training and were not provided with any
anchors on the firmness scale; individual consumers had to determine their own firmness classifications.

CONCLUSIONS

Researchers evaluating the texture of WPI gels do not need to take into account the effect of flavor on perceived textural attributes because the presence of flavor did not influence texture perception by either trained panelists or consumers. Furthermore, consumers were able to document and distinguish gel texture attributes in a similar manner to trained panelists. This research may have applications in evaluating gelled or chewy foods such as surimi, gelatin-based desserts or gummy/chewy candies. Further research in this area should evaluate the effect of flavor on other gel systems like agarose and carrageenan as well as investigate the effect of higher concentrations of flavor compounds on the perception of gel texture, while controlling for rheological differences.

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