EISSA, AHMED SHERIF. Enzymatic modification of whey protein gels at low pH (under the direction of Dr. Saad Khan)

Whey proteins are widely used in a variety of food products due to their functional and nutritional properties. This study focuses on modifying whey protein gel properties at acidic conditions by enzymatic treatment. Enzymes offer a powerful tool to modify biopolymers in general, and proteins in specific. Transglutaminase enzyme is used in this study to induce $\varepsilon-(\gamma$-glutamyl)lysine bonds between whey protein molecules at alkaline or neutral conditions, followed by subsequent acidification using glucono-$\delta$-lactone (GDL) to form gels at the desired acidic pH.

We examine the viability of $\beta$-lactoglobulin and $\alpha$-lactalbumin for crosslinking and conclude that $\alpha$-lactalbumin is easily crosslinked in its native state, while $\beta$-lactoglobulin needs partial or complete denaturation to undergo the enzymatic catalysis. Denaturation of $\beta$-lactoglobulin is done either by raising the pH to 8, or by thermal treatment (80 $^\circ$C for 1 hr) or by chemical denaturation using dithiothreitol (DTT). Crosslinks induced by transglutaminase increase the molecular weight of the whey proteins considerably to exceed $10^7$ Da.

In the first part of this study, we investigate the cold-set gel formation by initially conducting the enzymatic reaction at pH 8 and 50 $^\circ$C then following up with acidulation by GDL to pH 4. The resulting gel exhibits superior rheological properties with higher elastic modulus and substantially higher fracture/yield stress and strain compared to cold-set gels with no enzyme.
In the second part of this study, we examine an alternative route for crosslinking, in which we preheat the whey protein first at 80 °C for 1 hr at pH 7, and then conduct the crosslinking at 50 °C. This procedure induces both disulfide and ε-(γ-glutamyl)lysine bonds. The elastic modulus of the final gels shows a modest increase for samples treated with enzyme while the fracture strain and stress reveal significant increase with enzyme treatment. Interestingly, the microstructures of both gels, with and without enzyme treatment, are found to be similar with a fractal dimension of ~2 obtained independently using rheology and confocal microscopy. This suggests that the nonlinear fracture/yield properties are not reflected in the microstructure of the gels in the length scales probed using confocal microscopy.

In the third part of this study, we investigate the relative roles of physical and chemical interactions that affect the properties of protein polymers and cold-set gels at pH 4, prepared using the same protocol as in the second part of the thesis. We examine the role of hydrogen bonding, hydrophobic interactions, disulfide bonds and ε-(γ-glutamyl)lysine bonds. Physical interactions do not play a major role in the molecular weight or the size of the polymer, prior to acidification. However, they affect the gel rheological properties profoundly. The disruption of the hydrogen bonds inhibits gelation, while the disruption of the hydrophobic interactions causes extensive syneresis and result in weak and fragile gels. Chemical bonding affects the gel elastic modulus mildly and plays a detrimental role in the fracture/yield properties. The introduction of ε-(γ-glutamyl)lysine bonds increases the fracture strain and enhances the rheological properties of the gels that lack the disulfide bonds.
In the fourth part of the thesis, we investigate the conformational characteristics of β-lactoglobulin – the main constituent of whey proteins - subject to enzymatic crosslinking, using Fourier Transform Infrared (FTIR) spectroscopy. We find major differences between thermal denaturation and chemical denaturation (using DTT or β-mercaptoethanol). Crosslinking by transglutaminase of the thermally denatured β-lactoglobulin does not change the spectra in the amide I region but alters the C-H stretching mode, suggesting modification of the hydrophobic interactions. On the other hand, crosslinking by transglutaminase of the chemically denatured β-lactoglobulin changed the structure of the α-helix and induced intermolecular β-sheets.

In the fifth part of the study, we derive a transglutaminase-catalyzed polymerization model of β-lactoglobulin based on probabilistic approach of non-linear polymers. Derived equations show critical gelation conversion of 5.8%. Although the model is based on several assumptions, we believe it represents the starting point towards a more realistic model taking into account the intramolecular crosslinks and the unequal functionalities of the lysine and glutamine residues along the β-lactoglobulin backbone.

In the last part of the study, we present a brief discussion of the effect of transglutaminase on hydrophobic associations in chemically (using DTT) denatured whey proteins. Interestingly, we find that crosslinking of whey proteins by transglutaminase can modulate and screen the hydrophobic associations. This modulating effect is attributed to the compactness of the crosslinked protein chains that limit exposure of hydrophobic moieties to form hydrophobic associations.
ENZYMATIC MODIFICATION OF WHEY PROTEIN GELS AT LOW pH

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Chair of Advisory Committee
DEDICATION

To Allah (Lord) to whom all our deeds are dedicated.

To my mother, who brought me up and swarmed with me with love and compassion.

To my father, who taught me discipline and supported my scientific route in every possible means.

To my brother, the big heart and the wise mind, who is all the times there for me.

To my wife, who cares, gives, loves and helps me with not limit

To the two lights of my eyes, my two daughters, Salma and Aleya, who brought the true cheer to my life.
BIOGRAPHY

Ahmed Sherif Eissa was born in Cairo, Egypt in December 1st 1974. He joined the college of Engineering in Cairo University in 1992 and graduated in 1997 with a major in Chemical Engineering with honor degree. He was nominated to work in the same university as a teaching assistant. Ahmed served in the Egyptian Army from January 1998 to March 1999. While serving in the army he was working in parallel to finish his masters in the same school, studying gas-liquid mass transfer. He obtained his M.Sc. degree in Chemical Engineering in October 1999. Then he got married to his wife, Shakinaz ElSherbiny in June 2000, just two months before joining North Carolina State University as Ph.D. student in the Department of Chemical Engineering. Ahmed was blessed with two baby girls during his stay in the US, Salma in May 2001, and Aleya in October 2003. Upon finishing his graduate studies in NC State, he will be returning to his home school, Cairo University, as an assistant professor of Chemical Engineering.
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CHAPTER 1

INTRODUCTION AND OVERVIEW
ABSTRACT

In this chapter, we introduce the reader to the thesis topic - whey protein gels at low pH via enzymatic treatment. Many food products make use of whey proteins due to their abundance, excellent nutritional values and unique functional properties. Yet, efficient utilization of whey proteins requires modification to tailor their texture according to the specific food product. At low pH condition – which is the focus of this thesis – we can use enzyme to customize the rheological properties of the whey proteins to introduce richer and more consumer-appealing texture. In this regard, we are using transglutaminase enzyme to crosslink whey proteins to produce gels with enhanced rheological properties at low pH. A variety of experimental techniques are used to gain a fundamental understanding of the properties generated by enzymatically altering the whey proteins molecular architecture.
1.1 INTRODUCTION

Whey proteins are essential ingredients that are utilized in numerous food products. Their high nutritional values, unique functional properties, abundance, and low production cost render them desirable in a wide variety of products in the US market. US production of whey protein exceeded 1 billion lbs in the year 2003 (1). Examples of food products containing whey proteins include confectionaries, salad dressings, ice creams, cheeses, soups, and many other dairy-based products.

Whey proteins have superior nutritional qualities manifested in high values of net protein utilization (defined as percentage of retained protein intake), and protein efficiency ratio (defined as gain in body weight divided by weight of protein consumed). These values even exceed those of casein and milk protein (2). Of equal importance are the functional properties of whey proteins that participate in the texture of the food products. Gelation, emulsification, foaming and thickening are typical functionalities of whey proteins (3-6). These functionalities are a direct reflection of the chemistry, conformation, and molecular interactions of the whey protein molecules.

Gelation is of special importance in food products as it provides unique textural properties that gives pleasant mouth feel, and enables holding water and other ingredients in one matrix. For these purposes, whey protein gels have been studied extensively to examine their textural and rheological properties and to understand the effect of physical conditions on the gelation properties (7-11). Ionic strength, pH, and temperature play a major role in determining the rheological properties of the gels.
Whey protein gels at low pH (<4.5) have the advantages of superior shelf stability, and less stringent sterilization processes. However, the rheological properties of such gels are weak and brittle (12) due to the absence of the disulfide links at low pHs. These properties limit their use in food products. In this regard, we investigate the use of enzymes to induce chemical crosslinks between whey protein molecules, to strengthen the gel network, to tailor the rheological properties and restore the desirable texture.

Enzymes have been used frequently to crosslink food proteins. More specifically, transglutaminase (EC2.3.2.13) has been used to crosslink whey and milk proteins (13-20) as the enzyme links glutamine and lysine residues by catalyzing the acyl transfer reaction and producing ε-(γ-glutamyl)lysine bonds. Although crosslinking by transglutaminase has been studied for some proteins such as αs1-casein, κ-casein, β-casein, 11s and 7s soy globulins, crosslinking of whey proteins using transglutaminase is still a work in progress. Moreover, little attention has been given on modifying whey proteins gels at acidic conditions. The specificity of enzyme-catalyzed crosslinks combined with its ability to work at different (low) pH and low temperature makes this a viable route for developing whey protein gels. In this thesis, we examine various approaches of developing whey protein gels at low pH with favorable rheological properties using transglutaminase enzyme.

The broad objective of this thesis is to promote a fundamental understanding of the structure-property relationship ensuing as a result of enzymatic modification of whey proteins. More specifically, the following goals have been addressed:
1. Identifying key features of the enzymatic crosslinking of whey proteins by transglutaminase in terms of variables such as: pH, ionic strength, enzyme concentration and substrate (protein) concentration
2. Understanding the effect of the enzymatic crosslinking on the protein molecular properties such as: molecular weight, molecular size, and conformational characteristics
3. Investigating the rheological properties of the crosslinked whey proteins gels, specifically, the elastic and viscous moduli in the linear regime and the yield and fracture properties
4. Examining the gel microstructure by microscopy techniques (scanning electron microscopy and confocal microscopy)
5. Understanding the relative effect of each type of interactions (chemical vs physical) on the molecular and rheological properties of the protein gels.

1.2 THESIS OVERVIEW

The thesis chapters are organized as follows. Chapter 2 introduces some basic information about whey proteins and their gelation properties and reviews the crosslinking of whey proteins by transglutaminase. Chapter 3 explains the experimental techniques used throughout the research. In chapter 4, we investigate the crosslinking conditions, where we select slightly alkaline conditions to conduct the crosslinking, and then we compare the rheological properties of the whey protein gels with and without enzyme treatment. We also examine the microstructure of the gels using scanning electron microscopy. In chapter 5, we formulate a different approach to develop whey protein gels in which we preheat the whey protein for 1 hr at 80 °C at neutral conditions
prior to enzyme crosslinking. We investigate the molecular weight of the resulting polymers, and the rheological properties of the resulting gels. Microstructure and fractal properties are also examined by confocal microscopy. In chapter 6, we examine the effect of the different types of bonds/interactions on the rheological properties and microstructure of whey protein gel. We attempt to obtain an understanding of the relationship between the structure and properties of the gels. Chapter 7 provides an infrared spectroscopy study of the conformational characteristics of β-lactoglobulin, needed for successful crosslinking and to understand the changes occurring after crosslinking. In chapter 8, we propose a polymerization model for the crosslinking of β-lactoglobulin by transglutaminase using a probabilistic approach. In chapter 9 we discuss the modulating effect of transglutaminase enzyme on the hydrophobic interactions between denatured whey proteins molecules. Chapter 10 concludes the thesis and presents some future research recommendations.
1.3 REFERENCES


CHAPTER 2

LITERATURE REVIEW
ABSTRACT

In this chapter, we review selected literature of low pH whey protein gels and enzymatic crosslinking by transglutaminase. We first introduce basic information about the individual whey proteins and their molecular characteristics. We then discuss the gelation functionality of the whey proteins with a focus on cold set gelation. We also discuss low pH whey protein gels and their weak textural properties. Enzymatic crosslinking is then introduced as a means of protein modification. Finally, we discuss the molecular and catalyzing properties of transglutaminase enzyme with an emphasis on its usage in whey protein crosslinking.
2.1 INTRODUCTION

Whey proteins are important food ingredients that are used in a variety of food products. They possess excellent nutritional values and unique functional properties. Functional properties reflect on the texture, and hence acceptability and overall quality of the food products. Mouthfeel of a food product plays a major role in making it appealing to consumers. Numerous food products are primarily marketed based on the smoothness, crispness, crunchiness or spreadability. Consequently, whey proteins have been studied extensively to investigate their textural properties and tailor their rheological properties. Acidity (i.e. pH) as well as the salt content, in addition to the thermal treatment used in product preparation affect its texture and mouthfeel. Some of these conditions may be detrimental to the texture of whey protein. Scientists and engineers investigate different routes for remedying and modifying food texture. In this research, we focus on whey protein gels at low pH, which suffer from a weak and brittle texture (1,2). A viable route to modify the properties of whey protein gel is by using enzymes to induce chemical crosslinks and strengthen the gel network. In the ensuing sections, we discuss selected reviews of the enzymatic modification of whey proteins. We first introduce basic information about whey proteins, followed by a brief explanation of gelation mechanisms with emphasis on cold set gelation. Following this we explain the enzymatic modification of whey protein concentrating on one specific enzyme; transglutaminase.

2.2 WHEY PROTEINS

The term whey proteins represent the group of milk proteins that are left after casein precipitation at pH 4.6 and 20°C (3). Industrially, whey proteins are extracted from
the aqueous part left after cheese making. Table 1 shows the protein fractions isolated from cow’s milk (4). Whey proteins consist mainly of β-lactoglobulin (β-LG), α-lactalbumin (α-LA), serum albumin (SA), immunoglobulin (IG) and lactoferrin (LF). Typical percentage of each fraction of these proteins in the whey proteins mixture is illustrated in Figure 1. There are two main groups of whey: (1) Sweet whey: This is also known as cheese whey and is produced during cheese making, when rennet is used. Sweet whey contributes in many food products. The pH value of sweet whey can range between 5.2 and 6.7. (2) Sour whey: This can be acid whey, quark or cottage cheese whey and sour sweet whey. Acid whey, also known as casein whey, originates from the production of casein by means of lactic acid and hydrochloric acid. Lactic acid created through natural fermentation gives the whey a high acidity. The pH values of these types of whey range from 3.8 to 4.6. Generally, sweet whey powder is associated with lower amounts of minerals. The most prevalent methods of whey protein production are ultrafiltration and diafiltration (5). Then, powdered proteins are obtained by spray drying to obtain whey protein concentrates (WPC). Further purification by ion-exchange produces whey protein isolates (WPI). WPC contains about 35 - 80 % protein while WPI contains > 90 % protein (6).

Whey proteins have excellent nutritional values, and unique functional properties, and are being used in many food products. Examples of food products containing whey proteins as one of their ingredients include: ice cream, cheese, salad dressing, confectionaries, and wide variety of dairy products. Nutritional values of whey proteins have been evaluated and compared to other proteins. Table 2 shows values of biological values, protein digestibility, net protein utilization, protein efficiency ratios and protein
digestibility corrected amino acid score. Whey proteins possess unique functional properties such as gelation, water binding, emulsification, foaming and thickening properties. Such functional properties are a reflection of the chemistry of their molecules as well as the secondary, tertiary and quaternary structures. Extensive work has been done to explore the functional properties of whey proteins. In the next section, we discuss the properties of the main constituents of whey proteins.

2.2.1 COMPONENTS OF WHEY PROTEINS

2.2.1.1 β-LACTOGLOBULIN

β-Lactoglobulin (β-LG) is the major protein in whey and is considered a member of the lipocalin family of proteins (7). It occurs mainly in two genetic forms A and B. Both the A and B genetic variants occur at high frequency in most breeds of cow, and the presence of one or the other of these two variants affects the properties of the milk markedly (8,9). β-LG is composed of 162 amino acids and the molecular weight is 18,362 for genetic variant A and 18,276 for variant B (5). The isoelectric point of β-LG is ~ 5.13 (3). The amino acid sequence of β-LG is shown in Figure 3. β-LG contains five cysteine residues; four of them are involved in two intramolecular cystine crosslinks (Cys66-Cys160) and (Cys106-Cys109) and one is free (Cys121) (10-12). The free Cys121, with its reactive thiol group is involved in the denaturation and aggregation behavior of β-LG (13).

Under physiological conditions, β-LG exists as dimer. The secondary structure of β-LG under these conditions consist of nine β-strands (~50%), single 3-turn α-helix (10–15%), turns (~20%), and random arrangements (~15%) (14). The nine β-strands are eight stranded, antiparallel β-barrel with a ninth β-strand flanking the first strand. It is
this ninth strand that forms a significant part of the dimer interface in bovine β-LG. An important feature of bovine β-LG is the ability to bind hydrophobic and amphiphilic moieties (7). Binding of retinol (vitamin A) to β-LG has been extensively studied. Although some retinoids and fatty acids can bind in the deep hydrophobic pocket of β-LG (15,16) there is some doubt about the biological role of this protein. From a functionality standpoint, the behavior of β-LG is known to dominate whey protein ingredients, especially those with higher protein contents such as WPI and some WPC (17).

2.2.1.2 α-LACTALBUMIN

α-Lactalbumin (α-LA) is the second major constituent of whey protein. α-LA has two predominant genetic variants (A and B). α-LA has an isoelectric point of ~ 4.35 (3) and consists of 123 amino acids with a formula weight of 14,169. The amino acid sequence is shown in Figure 3. α-LA is an ideal protein for the nutrition of human infants (18). It contains of 8 cysteine resides. They are involved in 4 cystine intramolecular crosslinks (Cys6-Cys120), (Cys28-Cys111), (Cys61-Cys77) and (Cys73-Cys91). The whey protein α-LA has a specific and defined physiological function in the mammary gland. Within the Golgi apparatus of the mammary epithelial cell, α-LA interacts with the ubiquitously expressed enzyme β-1,4-galactosyltransferase to form the lactose synthase complex. α-LA modifies the substrate specificity of β-1,4-galactosyltransferase, allowing the formation of lactose from glucose and UDP-galactose.

Bovine α-LA and bovine lysozyme show a 62.6% similarity and 35.8% identity at the amino acid level. α-LA binds to calcium, zinc, and other metals. There is evidence that the binding of calcium is necessary for proper folding and disulfide bond formation of the native protein (19). α-LA has distinct stability properties as it adopts stable
conformational state known as “molten globule” under slightly denaturing conditions (20). The secondary structure of α-LA consist of α-helix (~31%), $3_{10}$-helix (~21%) and a small contribution of β-strands (~6%) (21).

2.2.1.3 SERUM ALBUMIN

Serum albumin (SA) represents about 8% of the total whey protein. It is a major blood serum protein and plays a major role in the transport, metabolism, and distribution of ligands, contributes to the osmotic pressure of blood (22) and imparts free radical protection. Bovine SA (BSA), as found in milk, is identical to blood SA (23,24). BSA consists of 585 amino acid residues with 17 disulfide bonds and a formula weight of 66,399 Da. The amino acid sequence of BSA is shown in Figure 4.

2.2.1.4 IMMUNOGLOBULIN

Immunoglobulin (IG) fraction accounts for about 1% of total milk protein or about 6% of total whey protein. Immunoglobulins occur as polymers or protomers of a basic “Y shaped” unit composed of 4 polypeptide chains linked by inter- and intramolecular disulfide bonds. The monomers consist of 2 identical heavy chains (H) with molecular weights ranging from about 55 to 76 kDa, depending on the class, and 2 identical light chains (L) with molecular weights of 22.5 to 27.3 kDa.

2.2.1.5 LACTOFERRIN

Lactoferrin (LF) is of mammary origin and can be found in the milk of most species (25). The typical concentration is between 20 and 200 µg/L. But the actual concentration increases noticeably in case of inflammation or infection. LF consists of 689 amino acids and has a formula weight of 75,758 Da. LF is a multifunctional protein and it is used in a variety of applications such as neutraceuticals in infant formulas, health
supplements, oral care products, and animal feeds to capitalize on its ability to boost natural defense against infections (26).

2.2.2 GELATION OF WHEY PROTEIN

The scientific term “gel” was first introduced by Thomas Graham (>1869). Since then, many definitions have been introduced to describe the gel state. Gelation involves the construction of a continuous network of macroscopic dimensions (27). Network formation requires denaturation of the protein molecules (28) followed by intermolecular interactions. Denaturation is required to expose the reactive functional groups of the protein. Interactions between these functional groups involve chemical bonding and physical interactions. Chemical bonding typically involves disulfide bonds formation, which is crucial for protein aggregation (29-32). Physical interactions include van der Waals interactions, hydrogen bonding, electrostatic and hydrophobic interactions (33). A minimum protein concentration is required for gelation. This concentration is a function of temperature, pH and ionic strength. Gelation is usually done via heat treatment at a temperature higher than denaturation temperature. However, if the protein concentration is less than the minimum concentration needed for the gelation, we obtain a soluble protein polymer (aggregates) (34). Soluble protein polymers can be gelled by changing the physical environment such as pH or ionic strength. This gelation is known as cold set gelation (35).

2.2.2.1 COLD SET GELATION

Obtaining gels at ambient of near ambient temperature is a general practice in food industry. Large molecular weight polymers, upon hydration, will interact with each other and with the solvent to produce gel or gel-like food product. A practical example is
the preparation of gelatin gel from gelatin powder. Cold gelation of whey proteins is typically done by changing the solvent quality of pre-denatured molecules (35), either by acidification or salt addition. Cold gelation of whey proteins has been a subject of numerous studies since the 1990s, with an emphasis on salt induced gelation rather than acid-induced gelation (36-42). In both cases the electrostatic interactions are screened and repulsion is minimized causing onset of gelation. Acidification is typically done by the addition of slow release acid such as glucono-δ-lactone (GDL) (43-45). As the pH decreases and approaches the isoelectric point of the protein, repulsion is minimized and gelation occurs. In the case of cold set gels by salt addition, NaCl or CaCl$_2$ are usually added to screen the electrostatic interactions.

Cold set gelation is advantageous when one or more of the ingredients are heat sensitive (46). Cold gelation produces gels with different microstructures than heat induced gels due to the different interactions involved in these gelation routes (35). Although salt-induced cold set gelation has been thoroughly addressed in literature, little is known about the acid-induced cold set gelation (35).

**2.2.3 WHEY PROTEIN GELS AT LOW pH**

Gelation of whey proteins at low pH may be thermally induced (47-49) or cold set (34,35), as we explained in the previous section. Thermally induced gelation at pH close to isoelectric point creates opaque gels with particulate microstructure, compared to transparent gels far from the isoelectric point. Several studies have been performed on gelling properties of whey proteins under acidic conditions (50-52). Water holding capacity was found to decrease noticeably as pH approaches the isoelectric point (47). Non-covalent interaction is thought to dominate the gel structure at pH $\leq$ 4 with no
disulfide bonds (47). Fracture stress and strain are also affected profoundly by the absence of disulfide bonds (2). Stading and Hermanson (1991) (1) studied β-LG gels at different pHs and found that gels formed at low pH (< 4) were brittle with low strain and stress at fracture, as opposed to those formed at high pH, which were rubber-like with high strain and stress at fracture. Errington and Foegeding (1998) (2) studied whey protein gels and found that gels formed at pH 3.0 and 2.5 were weak (fracture stress 17-19 kPa) and brittle (fracture strain 0.33 - 0.39) compared to fracture stress of 59-62 kPa and fracture strain of 1.2 - 1.2 at pH 7 and 6.5.

On the other hand, low pH environment is advantageous as it limits the microbial growth and increases the products shelf life (53). So, utilization of whey protein gels at acidic conditions will be beneficial in reducing spoilage, but the rheological properties have to be modified to overcome inherent weakness and brittleness of the gels. Enhancing the textural properties of whey protein gels at low pH is viable through acid-induced, cold-set gelation, in which disulfide bonds are created in a neutral pH and then the pH is dropped to the required acidic pH (2). An alternative route is to induce enzymatic crosslinks to strengthen the protein network and enhance the texture. This route has not been investigated in the literature and forms the focus of this research.

2.3 ENZYMES AS A TOOL TO MODIFY WHEY PROTEINS

Modification of whey proteins by enzymes can be done either by hydrolyzing enzymes or crosslinking enzymes. In hydrolyzing enzymes, protein molecules are cleaved into smaller peptides, producing what is known as Whey Protein Hydrolysates (WPH), which are known for their excellent nutritional values and functional properties (46). On the other hand, crosslinking enzymes, which are the focus of our research,
produce permanent bonds between whey protein molecules. The specificity of enzyme-catalyzed crosslinks combined with its ability to work at different (low) pH and low temperature makes this a viable route for developing whey protein gels. Several enzymes have been used to crosslink whey proteins such as polyphenol oxidase, tyrosinase, transaminase, transglutaminase, peroxidase, laccase, lipoxygenase, monoamine oxidase, protein disulfide isomerase, and sulfhydryl oxidase \((54-60)\). A thorough review of enzymatic crosslinking of food proteins is introduced by Matheis and Whitaker (1987) \((61)\). A more recent review by Schwenke (1997) \((62)\) discusses enzyme and chemical modifications of food proteins including progressive proteolysis, glycosylation, phosphorylation, acylation, deamidation, carboxyl esterification, and protein crosslinking. Crosslinking enzymes produce protein polymers and/or gel according to the reaction conditions.

One of the most widely used enzymes is transglutaminase. It has been used with different proteins such as \(\alpha_{s1}\)-casein, \(\kappa\)-casein, \(\beta\)-casein, 11s and 7s soy globulins, gelatin, ovalbumin, gliadin, glutenin, and whey proteins. In the following section we briefly describe the history, uses and molecular properties of transglutaminase and then discuss the previous work on crosslinking of whey proteins by transglutaminase.

2.3.1 TRANSGLUTAMINASE

The term transglutaminase (TG or Tgase) was first introduced by Clarke et al. in 1957 \((63)\) to describe the transamidating activity observed in guinea-pig liver. Studies on blood clotting \((64-67)\) revealed that enzymes catalyzed the stabilization of fibrin by cross-linking proteins through an acyl-transfer reaction between the \(\gamma\)-carboxamide group of peptide-bound glutamine and the \(\varepsilon\)-amino group of peptide-bound lysine, resulting in a
\(\varepsilon-(\gamma\text{-glutamyl})\text{lysine isopeptide bond} \) (Figure 5). Since this finding, proteins showing TG activity have now been found in wide variety of living matter such as micro-organisms, plants, invertebrates, amphibians, fish and birds. Mammalian transglutaminase requires the binding of \(\text{Ca}^{2+}\) for their activity, but at concentrations normally in the supraphysiological, not the physiological range associated with most intracellular processes. Moreover their \(\text{Ca}^{2+}\) activation is also modulated by further regulatory processes, which in essence means that they are virtually inactive under normal conditions and only activated following major disruptions in physiological homoeostatic mechanisms. Being a protein crosslinker, transglutaminase has been studied as a means to enhance the texture of different food proteins by inducing links between the protein molecules. Transglutaminase was used to crosslink \(\alpha_s1\text{-casein}, \kappa\text{-casein}, \beta\text{-casein}, 11s\) and \(7s\) soy globulins, gelatin, ovalbumin, gliadin, glutenin, and whey proteins. It is worth mentioning that transglutaminase has been used to crosslink wool fibers (68) and led to superior fibers properties such as increasing fabric strength and reduction of shrinkage.

Industrial production of transglutaminase from mammalian sources is not economically feasible; hence microorganisms were used to produce microbial transglutaminase. Transglutaminase was found in streptoverticillium sp. and streptomyces sp. The first production of microbial transglutaminase was reported by Motoki et al. (1989) (69,70) and Ando et al. (1989) (71). The molecular properties of the mammalian transglutaminase are different from that of the microbial transglutaminase. The most widely used microbial transglutaminase is that derived from \textit{streptoverticillium S-8112}. Microbial transglutaminase has a molecular weight of 37,849 Da and consists of 331 amino acids, as shown in Figure 6. A cysteine residue is located in the active center
of the enzyme. One of the major advantages of microbial transglutaminase is that its activity is independent of the calcium content. Table 3 shows the effect of calcium content on both microbial and guinea-pig liver transglutaminase. The SH group in the cysteine is involved in the catalytic reaction of transglutaminase. The activity of the enzyme is inhibited by reagents that modify SH-groups such as N-ethylmaleimide (NEM). The optimum pH for the enzyme is from ~ 6 to 7 and the optimum temperature is between ~50 and 55 °C (72). Activity of the enzyme is typically measured by what is known as the “hydroxamate method” (73). In this method, a primary amine (typically hydroxyl amine) is incorporated into a synthetic substrate, benzyloxy carbonyl-l-glutaminylglycine (Z-Gln-Gly). Then the quantity of hydroxamic acid produced is quantified by reacting with ferric chloride and tetra chloro acetic acid and measuring the resulting ferric complex at 525 nm.

2.4 CROSSLINKING OF WHEY PROTEINS BY TRANSGLUTAMINASE

Crosslinking of whey proteins using TG has been studied in the literature (54,58,60,74-81). Some of these studies examined the crosslinking of whey protein with other proteins such as casein (76,79), myofibrillar protein (82), and soy proteins (74) while other studies investigated the crosslinking of whey protein molecules themselves. Studies of whey proteins crosslinking showed difference in crosslinking susceptibility between the different whey proteins. β-LG, the main constituent of whey proteins, requires partial denaturation, while α-LA was readily susceptible to crosslinking. Aboumahmoud and Savello (1990) (60) studied the crosslinking of α-LA, β-LG and their blends by transglutaminase over a wide pH range (6.5 to 8.0). Faergemand et at (1997) (58) used transglutaminase to crosslink whey proteins and β-LG, and concluded that the
presence of reducing agent is required for the crosslinking of β-LG, while α-LA can be crosslinked in its native state. They also obtained gels at protein concentration higher than 10%. Dickinson and Yamamoto (1996) (83) studied the crosslinking of β-LG by transglutaminase and found that gels produced from transglutaminase crosslinking were less frequency dependant than thermally induced gels, indicating more solid-like behavior in case of crosslinking. Yildirim and Hettiarachchy (1998) (84) used transglutaminase to crosslink whey proteins forming films with higher solubility resistance compared with films with no transglutaminase treatment. Lauber et al. (2001) (77) studied the polymerization of β-LG by transglutaminase under high pressure as a means of protein denaturation. Truong et at. (2004) (85) studied the crosslinking of whey protein isolate by immobilized transglutaminase and found that crosslinked whey protein molecules exhibited higher viscosity and different gelation characteristics compared with non crosslinked whey protein molecules.

In the previously mentioned work, dithiotheritol (DTT) has been used to denature β-LG by cleaving the stabilizing disulfide bond in order to facilitate crosslinking (58,60). However, the use of such reagent is not allowed in food application. On the other hand, it is clearly noticed that cold set whey protein gelation using transglutaminase has not been addressed in the literature. In particular, the role of transglutaminase-induced bonds in fortifying the whey protein network at low pH has not been discussed.
2.5 REFERENCES


Figure 1. Typical percentages of whey proteins in bovine milk.

Bovine Whey Protein Composition

- $\beta$-LG (~65%)
- $\alpha$-LA (~25%)
- Others (~10%)
LIVTQTMKGL DIQKVAGTWY SLAMAASDIS LLDAQSAPLR VYVEELKPTP
EGDLEILLQK WENGECAKKK IIAEKTKIPA VFKIDALNEN KVLVLDTDYK
KYLLFCMENS AEPEQSLACQ CLVRTPEVDD EALEKFDKAL KALPMHIRLS
FNPTQLEEQC HI

**Figure 2.** Amino acid sequence of β-LG (variant A).
Figure 3. Amino acid sequence of α-LA (variant A).
Figure 4. Amino acid sequence of BSA.
**Figure 5.** Transglutaminase-catalyzed reactions. Reproduced from Zhu et al. (1995) (86)
Figure 6. Amino acid sequence of microbial transglutaminase.
**Figure 7.** Possible uses of transglutaminase. A single-lined oval in the middle indicates a protein to be modified, and double-lined ovals indicate substances to be incorporated. In this case, a protein to be modified is considered as the acyl acceptor. However, in some cases, it can be used as the acyl donor. Reproduced from (87)
### Table 1. Milk composition (4)

<table>
<thead>
<tr>
<th>Protein and Protein Fraction</th>
<th>Concentration in milk g/l</th>
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</thead>
<tbody>
<tr>
<td><strong>Total Proteins</strong></td>
<td></td>
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<tr>
<td>Casein</td>
<td>29.5</td>
</tr>
<tr>
<td>(\alpha_{S1})-Casein</td>
<td>11.9</td>
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<td>(\alpha_{S2})-Casein</td>
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</tr>
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<td>(\gamma)-Casein</td>
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<tr>
<td><strong>Whey Proteins</strong></td>
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<tr>
<td>(\beta)-lactoglobulin</td>
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<tr>
<td>(\alpha)-lactalbumin</td>
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</tr>
<tr>
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<td>Immunoglobulin</td>
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<tr>
<td>Protease-Peptones</td>
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Table 2. Qualities of different proteins. (88)

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<th></th>
<th>BV*</th>
<th>PD</th>
<th>NPU</th>
<th>PER</th>
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<tr>
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<td>95</td>
<td>86.45</td>
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<td>Casein</td>
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<td>100</td>
<td>76</td>
<td>2.9</td>
<td>1.23</td>
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<tr>
<td>Whey Protein</td>
<td>104</td>
<td>100</td>
<td>92</td>
<td>3.6</td>
<td>1.15</td>
</tr>
</tbody>
</table>

**Biological Value (BV)** = Proportion of absorbed protein that is retained in the body for maintenance and/or growth.

**Protein Digestibility (PD)** = Proportion of food protein absorbed.

**Net Protein Utilisation (NPU)** = Proportion of protein intake that is retained (calculated as BV x PD).

**Protein Efficiency Ratio (PER)** = Gain in body weight divided by weight of protein consumed.

**Protein Digestibility Corrected Amino Acid Score (PDCAAS)** = The amino acid score multiplied by a digestibility factor.

* The BV of egg protein is defined as 100
Table 3. Calcium dependency of microbial and Guinea-pig liver transglutaminases (72)

<table>
<thead>
<tr>
<th>CaCl₂ Concentration</th>
<th>Microbial Transglutaminase Activity</th>
<th>Guinea Pig Liver Transglutaminase Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mM</td>
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CHAPTER 3

EXPERIMENTAL TECHNIQUES
ABSTRACT

In this chapter we introduce brief description of the experimental techniques used to investigate the protein samples. These techniques include rheological characterization, gel permeation chromatography, reverse phase high pressure liquid chromatography, gel electrophoresis, confocal scanning laser microscopy, scanning electron microscopy and infrared spectroscopy. We explain the basic principles behind each of these techniques and comment on the importance of such techniques in exploring the properties of the tested materials.
3.1 INTRODUCTION

Exploration of the properties of food proteins in general and whey proteins in specific requires wide variety of experimentation. Rheological properties are of crucial importance, as they determine the texture, and hence the acceptability of the food product. We focused on the rheological properties in the linear viscoelastic region and at the fracture. We also used polyacrylamide gel electrophoresis and gel permeation chromatography to determine the molecular weight and molecular weight distribution of the protein polymers at different treatment conditions. We used gel electrophoresis under reducing and non reducing conditions to determine the extent of aggregation with and without disulfide bonding. In gel permeation chromatography we used static light scattering and infrared absorbance to determine the absolute the molecular weight of the protein aggregates with no need to molecular weight standards. On the microstructural level, we used two imaging techniques, laser scanning confocal microscopy, and electron scanning microscopy. These two techniques helped portraying a complete image of the protein microstructure. Confocal microscopy has the advantage of minimal sample alternation before imaging and also scans different sections through sample depth. On the other hand, scanning electron microscopy gives much higher magnifications up to 60,000x compared with 100x when using confocal microscopy. Finally, we used infrared spectroscopy to study the conformational characteristics of the protein molecules. Infrared spectroscopy is sensitive to the secondary structures of the protein such as α-helix and β-sheet structures.
3. 2 RHEOLOGICAL CHARACTERIZATION (1,2)

Rheology is defined as the science that deals with the deformation of materials as a result of an applied stress. It is a powerful tool that is capable of characterizing the properties and microstructure of many polymeric and non-polymeric systems. Rheology provides information that is important in designing and optimizing material properties. In the next few sections, we give a brief summary to different rheological techniques that have been used in this study.

3.2.1 STEADY-STATE OR SIMPLE SHEAR FLOW

In a steady shear experiment, a steady shear rate is applied to the sample and the resultant shear stress is measured. The apparent viscosity $\eta$ is defined as the ratio of the shear stress $\sigma$ to the shear rate $\gamma$ and reported as a function of the shear rate (or shear stress):

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

According to the behavior of $\eta$ as function of $\gamma$, the fluid can be classified as Newtonian (constant viscosity), pseudoplastic/shear thinning (viscosity decreases with shear rate), or dilatants / shear thickening (viscosity increases with shear rate). The different material responses are shown in Figure 3.

3.2.2 DYNAMIC OR SMALL AMPLITUDE OSCILLATORY SHEAR FLOW

The dynamic rheological technique is a useful tool in probing microstructures of materials without disrupting these structures. In dynamic (oscillatory) shear flow, a sinusoidally varying strain of amplitude $\gamma_o$ is applied to the sample:
\[ \gamma = \gamma_o \sin(\omega t), \text{ where } \omega \text{ is the frequency of oscillation.} \]

The dynamic experiment is usually carried out using very small strain amplitude and the sample is said to be within the linear viscoelastic (LVE) region (3). In the LVE region the sample response is independent of the applied strain amplitude and the stress generated due to the sinusoidal shear is also sinusoidal;

\[ \sigma = \sigma_o \sin(\omega t + \delta) \]

Here, \( \delta \) is the phase angle. For elastic solids both stress and strain are in phase \( (\delta = 0^\circ) \), while for Newtonian fluids, the stress and strain are completely out of phase \( (\delta = 90^\circ) \). Correspondingly, viscoelastic materials exhibit a phase angle between 0 and 90°. Using trigonometric identities, the stress wave can be decomposed into an in-phase and out-of-phase component: \( \sigma = \sigma_o \sin(\omega t) \cos(\delta) + \sigma_o \cos(\omega t) \sin(\delta) \)

The in-phase component corresponds to the ability of the material to elastically store energy and the out-of-phase component corresponds to its ability to dissipate energy. Moreover, the shear storage (elastic) modulus \( (G') \) and the shear loss (viscous) modulus \( (G'') \) is defined as the ratio of the corresponding stress component to the strain amplitude, as follows:

\[ G' = \frac{\sigma_o}{\gamma_o} \cos(\delta) \]
\[ G'' = \frac{\sigma_o}{\gamma_o} \sin(\delta) \]

The complex viscosity \( (\eta^*) \) is defined as

\[ \eta^* = \left( \frac{G'}{\omega} \right)^2 + \left( \frac{G''}{\omega} \right)^2 \right)^{0.5} \]
The frequency dependence of $G'$ and $G''$, also known as the dynamic mechanical spectrum, provides important information about the microstructure of a material. For example, gels exhibit $G'$ that is larger than $G''$ with both moduli independent of frequency. Polymer melts show $G'$ and $G''$ at low frequencies that are dependent on $\omega^2$ and $\omega$, respectively. For viscoelastic materials, the overlap frequency (the frequency at which $G'$ and $G''$ curves intersect) gives information about the relaxation time of the system. The plateau modulus, the value of $G'$ at high frequency, gives information about the strength of the structures formed in the material. Typical dynamic spectra for gels and common polymer solution or melts are shown in Figure 2.

Various geometries are used for rheological characterization of materials: cone and plate, parallel plates, and concentric cylinders. For insitu gelation, as in this work, concentric cylinders geometry is preferred. So, in all of our experiments we used the concentric cylinders geometry (schematic shown in Figure 3). When the inner cylinder rotates, the geometry is termed Searle geometry; however, when the outer cylinder rotates, it is termed Couette geometry.

### 3.3 LARGE STRAIN COMPRESSION TESTING (4)

Large strain compression testing aims primarily at investigating the failure of the materials under compression load. Typically, a Universal Testing Machine (Figure 4) is used to compress the sample up to certain compression ratio. There are three types of compression: uniaxial, biaxial and planer compression. However, uniaxial compression is the most widely used techniques to examine protein gels. In a uniaxial compression test, a force is applied to the longitudinal axis of the sample until failure occurs. Specimens are typically cylindrical and flattened on each end. This technique has two limitations which
must be considered: (i) it is difficult to apply a true axial load; and (ii) Friction between
the specimen and plate creates bending stresses and alters shape. However, the friction
between the specimen and the plate can be overcome by lubricating the top of the sample
using mineral oil.

The normal stress applied on the sample can simply be calculated by dividing the
normal force \( F \) by the specimen cross sectional area \( A \):

\[
\sigma_c = -\frac{F}{A}
\]

However, during compression, a change in the specimen geometry occurs and
hence the stress value is altered. This is corrected as follows:

\[
\sigma_c = -\frac{F}{A} \times \frac{L}{L_i}
\]

Where \( L_i \) is the initial specimen length and \( L \) is the length at any time.

For strain calculations, the true axial strain is calculated as follows:

\[
\varepsilon_c = \ln \left( \frac{L}{L_i} \right)
\]

It is important to emphasize that during compression testing, the specimens
experience not only compressive stresses and strains, but shear stresses and strains. The
following relationships may be derived from the compression tests for shear stress \( \sigma_s \) and
strain \( \gamma \).

\[
\sigma_s = -\frac{\sigma_c}{2}
\]

\[
\gamma = -1.5 \varepsilon_c
\]
During compression, the point of maximum torque is considered to be the onset of the fracture, and hence the stress and strain at fracture are extracted from this specific point.

3.4 GEL PERMEATION CHROMATOGRAPHY (5)

Gel permeation chromatography is a well established technique for macromolecules separation, through passage via column(s) filled with polymeric material matrix (also termed stationary phase). Large molecules elute earlier than smaller ones as the latter are not admitted to the small pores of the matrix and hence spend shorter time in the column(s) as shown in the schematic representation in Figure 5. Proteins of different molecular weight can be separated by gel permeation chromatography. Protein sample is injected to the system and carried out by the “eluent”, a mobile phase that is usually buffered at neutral or near neutral conditions. Ideally, there should be no interaction between the proteins (or the macromolecules in general) with the packing material (stationary phase) of the column. Most of the packing materials carry negative charge; hence, interaction between packing material and the negatively charged proteins will lead to repulsion and shorter elution time. On the other hand, interaction between the packing material and the positively charged proteins will lead to attraction and longer elution time. To ensure that there are no interactions between the proteins and stationary phase, the mobile phase has to be of high ionic strength, i.e., contains 0.1 – 0.3 M salt. The typical mobile phase used in the course of this work was: 10 mM Tris buffer, 0.1 M NaCl, and 0.02 % NaN₃ (to prevent microbial growth).
**Characterization of the separated proteins**

Separated proteins were characterized via triple detector system consisting of photodiode array detector (UV detector), interferometric refractometer (RI detector) and static light scattering detector.

Either the UV or RI detector is needed with the static light scattering to fully characterize the protein size and molecular weight, without any need for molecular weight standard. The following derivation shows the determination of molecular weight by the light scattering and RI detector.

For an incident light on polymer molecules, we have the following expression

\[
\frac{K^*c}{R(\theta)} = \frac{1}{M_w P(\theta)} + 2A_2c
\]

where \( c \) is the protein concentration, \( M_w \) is the weight-average molecular weight of the protein, \( A_2 \) is second virial coefficient of protein-water system, \( R(\theta) \) is the measured excess scattering intensity of the solution over that of the pure solvent, the Raleigh ratio, and \( P(\theta) \) is the particle scattering function as a function of angle relative to incident beam and is given by *

\[
\frac{1}{P(\theta)} = 1 + \left(\frac{4\pi}{\lambda}\right)^2 \sin^2 \left(\frac{\theta}{2}\right) \left(\frac{\langle R_g^2 \rangle_z}{3}\right) \quad \text{...................... (*)}
\]

where \( \lambda \) is the wavenumber of the incident light in the solution and \( \langle R_g^2 \rangle_z \) is the mean square radius of gyration of the molecules in solution.

\[
K^* = \frac{4\pi^2 n_o^2 (dn/dc)^2}{\lambda_o^2 N_A}
\]
Where \( n_o \) is the refractive index of the solvent = 1.33 for water, \( dn/dc \) is the specific refractive index increment of the solution \( \sim 0.19 \) for proteins, \( \lambda_o \) is wavelength of the incident light in vacuum, and \( N_A = \text{Avogadro’s number} \).

The RI detector is the means by which we determine the protein concentration. The weight-average molecular weight, the radius of gyration, and the second virial coefficient can be determined by measuring the scattered intensity as a function of angle for a series of different dilute concentrations. These parameters are determined from a Zimm plot \( K^*c/R(\theta) \) against \( \sin^2(\theta/2) + kc \), where \( k \) is an arbitrary constant used to spread out the data (Figure 6). The data are extrapolated to zero concentration, and the double extrapolation to zero angle and zero concentration intercepts the \( K^*c/R(\theta) \) axis at a value equal to inverse of the molecular weight.

\[
\left( \frac{K^*c}{R(\theta = 0)} \right)_{c \rightarrow 0} = \frac{1}{M_w}
\]

In the GPC with light scattering, the molecular weight is measured for each concentration, so the value of \( A_2 \) should be known in order to determine \( M_w \). It is usual practice to assume \( A_2 = 0 \) to determine \( M_w \). This assumption is usually valid since the eluting concentration is very low and hence the interactions are minimal. From the slope of the zero concentration line, we can get the radius of gyration. However, the value of radius of gyration is reliable only when it is larger than \( \sim 20 \text{ nm} \).

3.5 REVERSE PHASE HIGH PRESSURE LIQUID CHROMATOGRAPHY (RP-HPLC) (6,7)

In RP HPLC, compounds are separated based on their hydrophobic character. The stationary phase is non-polar while the mobile phase (eluent) is a linear gradient of
aqueous solution and organic solvent. The mobile phase is usually a mixture of water, methanol and acetonitrile. The peptides or amino acids are retained into RP HPLC columns in high aqueous mobile phase and are eluted from the columns with high organic mobile phase. The stationary phase is generally made up of hydrophobic alkyl chains (CH₂-CH₂-CH₂-CH₃) that interact with the peptides or amino acids. There are three common alkyl chain lengths, C₄, C₈, and C₁₈. C₄ is generally used for proteins and C₁₈ is generally used to capture peptides or small molecules. The idea here is that the larger protein molecule will likely have more hydrophobic moieties to interact with the column and thus a shorter chain length is more appropriate. Peptides and amino acids are smaller and need the more hydrophobic longer chain lengths to be captured; so C₈ and C₁₈ are used for peptides and amino acids. These alkyl chains are supported on the column packing material, typically silica particles. The beads or particles are generally characterized by particle and pore size. Particle sizes generally range between 3 and 50 microns, with 5 µm particles being the most popular for peptides. The particle pore size generally ranges between 100-1000 Å. Figure 7 shows a schematic representation of silica particles with alkyl chain attached to the surface in a typical separation process, where the less hydrophobic amino acids or peptides leave the column when the eluent is highly aqueous. Elution is done using two-solvent gradient, usually starting at near 100% aqueous and ramping to ~ 60% organic solvent in ~ 60 minutes. As the gradient keeps increasing the organic content, the more hydrophobic amino acids can leave the C₁₈ chains and elute with the organic solvent. Amino acids do not interact efficiently with the stationary phase due to their high polarity. So, they are usually chemically derivatized by adding highly hydrophobic moieties to the molecules to enhance their separation.
Examples of derivatizing agents are: o-phthalaldehyde (OPA), Phenylisothiocyanate (PITC) and butylisothiocyanate (BITC). In the work done in this research, we used PITC for derivatization.

3.6 GEL ELECTROPHORESIS (8)

Polyacrylamide gel electrophoresis (PAGE) is a protein separation technique based on the rate of migration in an electric field through a polyacrylamide gel. Protein samples are placed on highly cross-linked gel matrix of polyacrylamide and an electric field is applied. The matrix is buffered to a mildly alkaline pH so that most proteins are anionic and migrate toward the anode (the positive electrode). Samples are injected on the top of the gel and run at once, together with a reference sample, typically a molecular weight marker consisting of known molecular weight proteins. Proteins migrate through the gel matrix. Two forces are affecting the macromolecules, the resisting force of the gel polymers and the electrokinetic force of the migrating macromolecule. Smaller proteins migrate quickly while larger ones are retarded as they move in the electric field. Hence, proteins are fractionated on the basis of both charge and mass.

In order to separate on the basis of mass only, a modification of the standard electrophoresis technique uses the negatively charged detergent sodium dodecyl sulfate (SDS) to overwhelm the native charge on proteins. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) is used to assess the purity and to estimate the molecular weight of a protein. In SDS-PAGE, the detergent is added to the polyacrylamide gel as well as to the protein samples. The dodecyl sulfate anion, which has a long hydrophobic tail \((\text{CH}_3(\text{CH}_2)_{11}\text{OSO}_3^-)\), binds to hydrophobic side chains of amino acid residues in the polypeptide chain. SDS binds at a ratio of approximately one molecule for every two
residues of a typical protein. Since larger proteins bind proportionately more SDS, the charge-to-mass ratios of all treated proteins are approximately the same. All the SDS-protein complexes are highly negatively charged and move toward the anode, as shown in Figure 8. However, their rate of migration through the gel is inversely proportional to the logarithm of their mass - larger proteins encounter more resistance and therefore migrate more slowly than smaller proteins. This sieving effect differs from gel-filtration chromatography because in gel filtration larger molecules are excluded from the pores of the gel, so the largest proteins travel most slowly. A reducing agent can also be added to the samples to reduce any disulfide bonds, and in this case, the system is said to be run “under reducing conditions”. The protein bands that result from this differential migration, schematized in Figure 8b, can be visualized by staining. Molecular weights of unknown proteins can be estimated by comparing their migration to the migration of reference proteins electrophoresed on the same gel.

3.7 LASER SCANNING CONFOCAL MICROSCOPY (LSCM) (9,10)

Laser scanning confocal microscopy (LSCM) is an imaging technique that probes the microstructure of wide variety of materials to determine their morphologies. It has an advantage of minimal sample disturbance or treatment as compared to other techniques scanning and transmission electron microscopy. The principle of LSCM is derived from conventional light microscope but in this case a laser in place of a light source is used. In addition, it has sensitive photomultiplier detectors and a computer set-up, which is used control various aspects of this technique. LSCM has been established as an important tool for obtaining high-resolution images and three-dimensional images of variety of specimens. A schematic diagram of a LSCM is shown in Figure 9. The operation
involves an x-y deflection mechanism, where the laser beam is turned into a scanning beam. It is then focused onto a small spot by an objective lens on a fluorescent specimen. The objective lens is used to capture a mixture of reflected and emitted fluorescent light. The diachronic mirror deviates the reflected light while the emitted fluorescent light passes in the direction of the photomultiplier. In front of the photodetector is a confocal aperture, which serves to obstruct the so-called out-of-focus information. This is especially important when dealing with thick specimens.

A 2-D image can be generated from a partial volume of sample centered at the focal plane. In addition, a series of images can be obtained at successive planes by alternating the focal plane in the z-direction. Using computer graphics, 3-D reconstructed images can be obtained which enable users to visualize structural details in a thin slice of a sample. Specimen to be imaged with a LSCM must be labeled with a fluorescent probe. Major factors to be considered when selecting a fluorescence dye are excitation/emission wavelengths of the probe, laser lines (wavelength of emission) available and the filter sets used. In our experimental work, we used Rhodamine-B as the fluorescence dye.

3.8 SCANNING ELECTRON MICROSCOPY (SEM) (11,12)

Scanning electron microscopy (SEM) is an imaging technique that examines, with large magnification, the microstructure of a wide variety of materials to reveal detailed morphologies. SEM utilizes an electron gun combined with a set of electrostatic and electromagnetic lenses to form and focus an electron probe onto a specimen surface. As the electrons bombard the sample material, they collide with atoms in the solid, thereby undergoing both elastic and inelastic scattering. Elastic scattering is defined as a change in trajectory without a loss of kinetic energy (see Figure 10). Inelastic scattering occurs
when a beam electron continues along its path but decelerates upon interaction with atoms or other electrons. The two main types of SEM signals used for imaging include back-scattered electrons (BSE) and secondary electrons (SE). Backscattered electrons are electrons with high energy (kV) from the incident electron beam that travel through the sample and reemit from the surface. Secondary electrons are electrons of low energy (<50 eV) that are generated from atoms in the material. For example, when a high-energy electron from the primary beam bombards an atom, it can eject a loosely bound outer shell electron or cause inner-shell ionization, which may lead to fluorescence or the ejection of an Auger electron. Both BSE and SE are emitted from the sample, and may be collected using a detector placed strategically within the sample chamber, at an angle above the sample surface. One of the most common detectors used today is an Everhart-Thornley detector, which consists of a biased shield for electron collection, a scintillator to convert electrons to photons, and a photomultiplier tube (PMT) to carry the photons to the cathode ray tube, or other type of display. The detector may be set to a positive bias (+300V) for pure BSE mode (no secondary electrons will be detected because of their low energy), or a negative bias (-100V) for detection of both SE and BSE. Backscattered and secondary electron images yield different types of information.

All of the imaging in this work was performed using the SE mode. One major problem that may be encountered when imaging with SEM is a phenomenon called charging. This occurs in non conducting materials, such as our whey protein samples, where electrons entering the specimen cannot be sufficiently conducted out of the sample material. This causes areas of the surface to build up a negative charge, so that drifting white spots appear on the image. The specimen may also be damaged from charging
effects. In order to avoid this problem, it is necessary to sputter coat the samples, typically with gold/palladium. The following steps were followed for preparation of whey proteins samples for SEM examination: (a) whey protein gels are cut into small pieces, (b) gels were fixed with 2.5% (w/w) glutaraldehyde in water for 1 hr and rinsed with water 3 times, (c) gels are then soaked in 0.2% (w/w) osmium tetraoxide for 12 hr, (d) the gels were then dehydrated using a graded ethanol series and completely dried using critical point drying with CO$_2$, and (e) the dried samples were gently fractured and coated with Au/Pd by diode sputter coating prior to scanning.

3.9 INFRARED (IR) SPECTROSCOPY (5,13-15)

Infrared spectroscopy is a powerful technique that probes the molecular vibrations in polymers. The main idea behind IR spectroscopy is the interference between two beams to yield an interferogram, which is a signal produced as a function of the change of path length between the two beams. An oscillating dipole in the polymer (in our case, the protein) causes absorption of the radiation that has the exact same frequency of the oscillation of the dipole moment. Figure 11 shows the basic components of IR spectrometer. There are different types of interferometers; however, the most commonly used interferometer is Michelson interferometer. In this interferometer, a beam splitter (of semi transparent material) splits into two beams of equal radiation, each beam falling on one mirror. The two mirrors are in two perpendicular planes. One of them can travel perpendicularly to its plane. The two beams are reflected by the mirrors and return to the beam splitter where they recombine and interfere (see the schematic in Figure 12).

The absorption bands of the amide groups in proteins are similar to that absorption band of secondary amides in general. There are nine amide bands in proteins: Amide A, Amide
B, Amides I-VII in order of decreasing wavenumber. The most useful amide band is Amide I which consists of 80% C=O stretching, 10% C-N stretching and 10% N-H bending. Amide I takes place in the infrared spectrum of 1600 to 1700 cm\(^{-1}\). Extensive intermolecular and intramolecular hydrogen bonding occurs between the N-H group and C=O in the proteins. The difference patterns of hydrogen bonds determines the relative arrangements of C=O groups and the degree of dipole-dipole interactions. Hence, studying the C=O stretching mode reflects the hydrogen bonds associated with the protein secondary structures. Accordingly, the Amide I region is a composite of different overlapping elements corresponding to helices, \(\beta\)-sheets, turns and random structures. In order to separate these elements, deconvolution mathematical tools are used, most widely Fourier Transform.

Another region of interest is the C-H stretching mode, typically in the spectral range of 2800 - 3100 cm\(^{-1}\) corresponding to diversity of CH, CH\(_2\), and CH\(_3\) in the side chains of the amino acids. The main band at 2940 cm\(^{-1}\) for protein was reported to represent aromatic, aliphatic and other amino acids. Bands around 2900 cm\(^{-1}\) and 2880 cm\(^{-1}\) primarily represent aliphatic amino acids. This region reflects the hydrophobic interactions between the protein molecules. Through the experimental work in this research we examined the Amide I region as well as the C-H stretching mode region.
3.10 REFERENCES


Figure 1. The behavior of Newtonian, shear thinning, and shear thickening materials under steady shear experiments.
Figure 2. The behavior of a polymer solution in contrast to gel material under dynamic frequency sweep (mechanical spectrum) experiments.
Figure 3. Schematic of concentric cylinders geometry.
Figure 4. Mechanical details of a typical Universal Testing Machine.
Figure 5. Schematic representation of the molecular separation by gel permeation chromatography.
Figure 6. Molecular weight determination via Zimm plot. Radius of gyration is obtained from the slope at zero concentration.
Derivatized amino acids with different hydrophobicity

\[ C_{18} \text{ Alkyl Chains} \]

Aqueous Eluent

70% Aqueous, 30% Organic

40% Aqueous, 60% Organic

Figure 7. Schematic representation of amino acids separation by reverse phase HPLC.
Figure 8. Schematic representation of protein separation by gel electrophoresis (8).
Figure 9. Schematic representation of the scanning confocal laser microscopy (10).
**Figure 10.** Schematic of (a) SEM apparatus with (b) illustration of electron detection (12).
Figure 11. Basic component of infrared spectrometer.
Figure 12. Schematic of Michelson Interferometer (15).
CHAPTER 4
POLYMERIZATION AND GELATION OF WHEY PROTEIN ISOLATE AT LOW pH USING TRANSGLUTAMINASE ENZYME

Chapter 4 is essentially a manuscript by Ahmed Eissa, Satisha Bisram, and Saad Khan published in the *Journal of Agricultural and Food Chemistry*, Vol 52, PP 4456-4464, 2004
Polymerization and Gelation of Whey Protein Isolates at Low pH Using Transglutaminase Enzyme

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ABSTRACT

Dynamic and steady shear rheology is used to examine the synthesis of low-pH (~4) whey protein gels using a two-step process. The first step involves crosslinking of whey proteins at pH 8 and 50 °C using transglutaminase enzyme, while the second step entails cold-set acidification of the resulting solution using glucono-δ-lactone (GDL) acid. During the first step, the sample undergoes enzyme catalyzed ε-(γ−glutamyl)lysine bond formation with a substantial increase in viscosity. Acidification in the second step using GDL acid leads to a rapid decrease in pH with a concomitant increase in the elastic (G′) and viscous (G′′) moduli, and formation of a gelled network. We examine the large strain behavior of the gel samples using a relative new approach that entails plotting the product of elastic modulus and strain (G′γ) as a function of increasing dynamic strain and looking for a maximum, which corresponds to the yield or fracture point. We find the enzyme catalyzed gels to have significantly higher yield/fracture stress and strain compared to cold set gels prepared without enzyme or conventional heat set gels. In addition, the elastic modulus of the enzyme-catalyzed gel is also higher than its non-enzyme treated counterpart. These results are discussed in terms of the gel microstructure and the role played by the enzyme-induced crosslinks.

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4.1 INTRODUCTION

Whey proteins are important food ingredients that are used in a number of food products that includes dairy, confectionary, and dessert items. A major co-product of the cheese industry, whey protein production (dry) has been exceeding one billion pounds annually in the U.S. in recent years (1). In addition to their nutritional values, incorporation of whey proteins in food products can promote emulsification, stabilization, foaming and gelation properties. In this regard, whey protein gelation is of considerable interest because it can provide new food products with unique functional performance and favorable textural properties (2-4). Moreover, food gelling systems are potential alternatives in replacing fats in foods (5). Whey protein gels can substitute the role of fats in enhancing textural properties of foods. Such gels are most commonly produced using heat treatment (2;3;6-10) although other factors such as salt addition (11), acidification (12;13), and enzyme treatment (14-19) can cause gelation of whey proteins with or without heating. In many food applications, however, it is not desirable to heat the products to high temperatures to induce gelation, and it becomes advantageous to induce gelation at ambient or near ambient temperatures (20).

Whey protein gels, prepared by heating acidic whey protein solution (pH < 4.6) are weak and brittle (21) limiting their use in food products under such conditions. Such poor rheological properties occur mainly due to the absence of the strong disulfide bonds at these acidic conditions and pH-associated effects on the denaturation and aggregation reactions (21). However, disulfide bonds can presumably be introduced through a two-step procedure that involves creating chemical crosslinks at neutral pH by heating the
whey protein solution (step 1), followed by acidifying the polymerized protein solution to the required pH (step 2) \((21)\).

A powerful and alternative approach for producing permanent bonds in whey proteins is by using enzymes \((14;17;22;23)\). The specificity of enzyme-catalyzed crosslinks combined with its ability to work at different (low) pH and low temperature makes this a viable route for developing whey protein gels. In this regard, earlier work in our laboratory focused on direct crosslinking of whey proteins at low pH. Burke et al \((23)\) treated whey proteins at pH 4 with transaminase using a combined enzyme and heat treatment protocol (incubation with enzyme at 40 °C for 40 minutes, followed by incubation at 80 °C for 20 minutes). The resulting gels exhibited higher elasticity and strength than that produced through conventional treatment. In the present study, we take a different approach to producing whey protein gels at low pH using transglutaminase enzyme. Transglutaminase (TG, EC2.3.2.13), which has been used to crosslink whey protein \((14;17;23-27)\), links glutamine and lysine residues as it catalyses the acyl transfer reaction and produces \(\varepsilon-(\gamma\text{-glutamyl})\)lysine bonds. Although crosslinking by TG has been studied for some proteins such as \(\alpha_{s1}\)-casein, \(\kappa\)-casein, \(\beta\)-casein, 11s and 7s soy globulins \((28-36)\), crosslinking of whey proteins using TG is still not well developed. Most of the previous work used denaturant (such as dithiotheritol) to denature the protein in order to facilitate crosslinking \((15;27;37;38)\) However, the use of such reagents is not allowed in food application. Moreover, the cold set whey protein gelation using TG has not been studied. In particular, the role of TG-induced bonds in fortifying the whey protein network at low pH has not been discussed.
In this study, we adopt a two-step strategy to develop whey protein gels, using enzymatic treatment at alkaline conditions followed by cold setting using glucono-δ-lactone acid to low pH (4). While a two-step procedure has previously been used (21), it has been for heat-treated samples at high temperatures and without any enzymes that can lead to new bond formation and microstructure. The distinctive feature of our work involves the combined use of enzyme (TG) with cold setting conditions, monitoring the initial enzyme-catalyzed polymerization step both rheologically and biochemically, and evaluating the gelation characteristics in-depth. In this regard, we use a new dynamic rheological approach to explore the large strain behavior of samples and identify yield/fracture points. We investigate the different conditions affecting crosslinking of whey proteins by TG. Gels produced by enzymatic treatment at alkaline conditions followed by cold setting using GDL acid to low pH are compared to gels produced with no enzyme but cold set and those prepared with conventional heat treatment. Our results reveal enzymatic treatment to produce gels with superior rheological properties. These results are discussed in terms of the microstructure and role of enzyme-induced crosslinks in the gel network.

4.2 MATERIALS AND METHODS

4.2.1 MATERIALS

Whey protein isolate (WPI) were obtained from Davisco Food International (LeSueur, MN) and used as received. A commercial version of Transglutaminase enzyme (1 % enzyme and 99 % maltodextrin, by weight) was provided by Ajinomoto Co., Japan. Enzyme activity was determined using the hydroxamate method as described by Folk (39). The activity unit is defined as the amount of the enzyme producing 1μmole of
hydroxamic acid per minute at 37 °C. Gloco-no-δ-Lactone (GDL), N-ethylmaleimide was purchased from Fisher Scientific (Pittsburg, PA). Sodium Chloride (> 99%) were purchased from Sigma Chemicals (St.Louis, MO). De-ionized water (> 15MΩ) was used in all the experiments.

4.2.2 SAMPLE PREPARATION

Protein solutions were prepared by dissolving WPI powder in de-ionized water to obtain a final concentration of 7.5 % w/w, and stirring for about 1 hr to ensure complete solubility. The pH was adjusted by using either NaOH (1 N) or HCl (1 and 0.1 N). Solutions were de-aerated for about 15 minutes under vacuum of 25 inch Hg at room temperature to eliminate trapped air bubbles.

Samples with enzyme were prepared by adding requisite amount of powdered enzyme to WPI solutions contained in vials, and mixing for 20 minutes at room temperature. The vials were closed immediately after enzyme addition to prevent enzyme deactivation through air-enzyme contact. Samples were then incubated for 5 hrs at 50 °C to induce enzymatic reaction. Samples without enzyme were heated at 50 °C for 5 hrs to mimic the thermal treatment of the samples with enzyme. N-ethylmaleimide (NEM) was added to some samples to prevent disulfide bonding.

To study the effect of ionic strength on crosslinking of protein by enzyme, NaCl was dissolved in the WPI solution at pH 8 before enzyme treatment. The molar concentration of NaCl ranged from 0 to 200 mM.

WPI gels at pH 4 were obtained by adding 1.85% GDL to the WPI solution (no salt added, originally at pH 8) at 25 °C and stirring for about 10 minutes. The solution was then poured into a rheometer cup. All rheological measurements were conducted at
25 °C, unless otherwise mentioned. Conventional heat-treated gels were obtained by heating WPI solution (7.5 % w/w, pH 4) at 80 °C for 5 hrs.

4.2.3 GEL ELECTROPHORESIS

Polyacrylamide gel electrophoresis (PAGE) under reducing conditions was performed on whey protein samples using Mini-PROTEAN 3 Electrophoresis Cell, (BIO-RAD, Hercules, CA) to obtain information on their molecular weights. Protein solutions (7.5%) were diluted 40 times (i.e., ratio of protein to buffer was 1:40) by Laemelli sample buffer (25% glycerol, 2% SDS and 0.01% Bromophenol Blue), mixed with 50 µl β-mercaptoethanol/ml of solution and heated for 5 min at 95 °C, and then subjected to electrophoresis in gradient gels of 4-15% polyacrylamide using the discontinuous system. These gels were stained with 0.1% Coomassie blue and destained using first a solution of high-destaining power (40 wt.% ethanol and 10 wt.% acetic acid in de-ionized water) for 2 hrs, and later a solution of low-destaining power (10 wt.% ethanol and 10 wt.% acetic acid in de-ionized water) for approximately 12 hours. The gels were subsequently immersed in a drying solution (containing 20% ethanol and 10% glycerol in de-ionized water) for 30 minutes, and fixed in drying frames for 24 hrs. The dry gels were finally examined to determine the different molecular weight bands. Gels under non-reducing condition was prepared the same way as above but without the addition of β-mercaptoethanol.

4.2.4 RHEOLOGICAL MEASUREMENT

Steady and dynamic shear rheological experiments were conducted on a TA Instruments AR2000 rheometer using a couette geometry (cup diameter = 30mm and bob diameter = 28mm). In the steady shear experiments, samples were heated to 50 °C and
maintained at this temperature. Viscosity was measured at 5-minute intervals while subjecting the sample to a continuous shear rate of 50 s\(^{-1}\). In the dynamic experiments, the samples were subjected to a sinusoidal deformation as either a function of increasing stress amplitude or frequency of oscillation, and the corresponding elastic (G’) and viscous (G”) moduli were measured. The strain sweep experiments, which were conducted at a constant frequency of 1 rad/s, served two purposes. First, it provided the limit of linear viscoelasticity (LVE) that could be used in the frequency experiments. Secondly, as discussed later, it provided a method to examine yield and fracture stress/strain of the samples (41;42). The frequency spectrum of the elastic and viscous modulus, on the other hand, provided a signature of the state (e.g., liquid or gel) of the samples (43). In all experiments conducted, the samples were covered with n-hexadecane to prevent evaporation.

**4.2.5 FIELD EMISSION SCANNING ELECTRON MICROSCOPY (FESEM)**

FESEM (Jeol 6400F) was used with accelerating voltage of 5 kV, magnification of 10,000X to investigate microstructure of whey protein gels obtained under different conditions. Small pieces of gels were fixed with 2.5% (w/w) glutaraldehyde in water for 1 hr and rinsed with water 3 times. The samples were then soaked in 0.2% (w/w) osmium tetraoxide for 12 hrs. The gels were initially dehydrated using a graded ethanol series (44) and completely dried using critical point drying with CO\(_2\) (44). The dried samples were gently fractured and coated with Au/Pd by diode sputter coating prior to scanning.
4.3 RESULTS AND DISCUSSION

4.3.1 ENZYME ACTIVITY AT DIFFERENT pH

In order to develop the two-step gelation process, it is essential to (i) determine the conditions in which the enzyme will have sufficient activity, and subsequently, (ii) examine which of these conditions are most conducive for the enzyme-catalyzed polymerization of whey protein. In this regard, we first explored the effect of pH on enzyme activity at 50°C (Figure 1). We chose this temperature as it had been found to be the optimal temperature for transglutaminase use (Ajinomoto Data Sheet). From Figure 1, which shows relative activity as a function of time, it is apparent that enzyme activity decays rapidly for pH <5 or > 8. This behavior can be explained in terms of protonation and deprotonation of enzyme active site (cysteine) at low and high pH, respectively (45) or due to disruption of the active site conformation resulting from enzyme denaturation. We also find enzyme activity to be fairly stable for pH 5-7 and to have lower degree of stability at pH 8. Based on these results, we undertook enzymatic treatment of WPI samples at pH 6, 7, and 8, as detailed in the next section. Studies of enzymatic polymerization of WPI at pH 4 and 9 were not undertaken because of the rapid decay of enzyme activity under these conditions. Enzymatic polymerization at pH 5 was also excluded because native (not subjected to any treatment) whey protein precipitates out of solution immediately after the solution pH is lowered to this value.

4.3.2 WHEY PROTEIN POLYMERIZATION WITH ENZYME

The effect of pH on enzyme treatment of whey proteins were examined by incubating samples at 50 ºC for 5 hrs with 100 U enzyme/g protein at pH 6, 7, and 8. Figure 2 shows molecular weight of various samples following enzyme treatment
obtained using reducing SDS-PAGE analysis. In Figure 2a, the two bands appearing on the lowest (bottom) part of the gel correspond to the molecular weights of α-lactalbumin and β-lactoglobulin, as evident from the SDS-PAGE analysis of these individual components (Figure 2b). We find the β-lactoglobulin band to remain effectively unchanged upon enzyme treatment at pH 6 and 7, but to become quite faint at pH 8. On the other hand, the α-lactalbumin band disappears at all pH following enzyme treatment. These results indicate that α-lactalbumin can be readily crosslinked (polymerized) at different pH, consistent with earlier reports (15;46). However, β-lactoglobulin requires slightly alkaline medium to be crosslinked. The difference in behavior between the two proteins can be attributed to the molten globular state of α-lactalbumin that facilitates crosslinking at all pH due the disordered tertiary structure (26). In contrast, β-lactoglobulin is believed to get partially denatured at a high pH of 8, which then facilitates its crosslinking by the enzyme (47). To study the individual behavior of α-lactalbumin and β-lactoglobulin, we incubated pure fractions of α-lactalbumin and β-lactoglobulin with enzyme (Figure 2b). We can clearly observe that at pH 8, β-lactoglobulin molecules are crosslinked with each other in the absence of α-lactalbumin (lane 9). We can also observe from the same figure that pure fractions of α-lactalbumin are able to polymerize irrespective of the pH (lanes 3,4 and 5), whereas β-lactoglobulin needs alkaline pH to polymerize. Based on these results, pH 8 represents a viable environment for the crosslinking of whey proteins by Transglutaminase. Any enzymatic treatment reported in the rest of this study has therefore been conducted at a pH of 8.

To examine the relative portion of enzyme versus disulfide linkages formed when whey proteins are treated at 50 °C for five hours, SDS-PAGE analysis was undertaken for
both reducing and non-reducing conditions. Figure 2c shows these results for whey protein samples (pH 8) subjected to various types of treatment. Lane 2, which corresponds to native WPI, reveals that there is almost no polymerization occurring. This indicates that although whey proteins are partially denatured, heat treatment is needed to initiate polymerization. Treatment of WPI at 80 °C for 1hr (lane 3) reveals extensive polymerization via disulfide bonding (note the effect of reducing conditions in decreasing the molecular weight in lane 4). Heating the sample at 50 °C for 5hrs (lane 5) produces a weak high molecular weight band and some aggregates with molecular weight less than 93kDa (i.e., tetramers and lower). In contrast, presence of enzyme (lane 7) under similar conditions, reveal large proportion of high molecular weight aggregates that remain unabated even under reducing conditions (pH 8). This suggests that disulfide bonds forms in small quantity at pH 8 at 50 °C, while the overwhelming aggregation occurs via the enzyme-catalyzed ε-(γ-glutamyl)lysine crosslinking.

The effects of enzyme concentration and solution ionic strength on whey protein polymerization are displayed in Figure 3. SDS-PAGE analysis following incubation with different concentrations of enzyme at pH 8 and 50 °C for five hours is shown in Figure 3a. We find no further effect on polymerization for enzyme concentrations higher than 10 U/gm protein. This can be surmised from the constant intensity of the monomeric bands of α-lactalbumin and β-lactoglobulin from 10 units up to 100 units (lanes 5 through 10). Figure 3b shows the effect of salt concentration on the extent of polymerization. These samples (pH 8) were incubated with enzyme for 5 hrs at 50 °C. It is apparent that an increase in ionic strength decreases the extent of polymerization of whey protein chains. At larger salt concentrations, darker (or heavier) bands of α-lactalbumin and β-
lactoglobulin, indicating higher concentration of unpolymerized fraction of protein, is noticed. This behavior can be explained on the basis of electrostatic interactions. At higher ionic strength, these interactions between protein chains are screened and the chains collapse. This collapse prevents the “induced fit” required for enzyme action. According to the induced fit concept, the substrates (α-lactalbumin and β-lactoglobulin in our case) cause the three-dimensional structure of the enzyme to change. This change in the structure brings the catalytic groups (glutamine and lysine) into proper alignment and induce the (ε-(γ−glutamyl)lysine) bonds. The collapsed chains of the α-lactalbumin and β-lactoglobulin molecules at high ionic strength fail to produce the proper enzyme conformation.

To investigate the effect of the enzyme treatment on rheological properties, steady shear viscosity was measured at a fixed shear rate (50 s⁻¹) as a function of incubation time (Figure 4). No increase in viscosity is observed when incubating the enzyme with the whey protein solution at 50 °C at pH 6 or pH 7. This indicates that the few cross-links (mainly between α-lactalbumin molecules) have little effect on solution viscosity. On the other hand, there is an appreciable increase in viscosity at pH 8 due to the polymerization of both β-lactoglobulin and α-lactalbumin molecules, consistent with our SDS page analysis. It should be noted that heating whey protein at 50 °C and pH 8 induces some disulfide formation. This is also exhibited in Figure 4 in which we observe a modest increase in viscosity for a sample containing no enzyme.

4.3.3 COLD-SET ACIDIC GELS FOLLOWING ENZYME TREATMENT

After enzyme treatment at pH 8, the protein solution was acidified by GDL (1.85% by weight), a slow release food grade acidulant, to pH 4. The use of GDL over
other strong acids (e.g., HCl) thereby allowed the time scale of sample pH change to be long enough to produce homogenous gelation throughout the sample. Please note that minimal, if any, enzyme crosslinking occurred during acidification as the enzyme had negligible activity (Figure 1) following the initial polymerization step. Figure 5a shows change in protein pH following GDL addition. We observe two distinct regimes consisting of a large initial decrease in pH to approximately 4.7 in about 2hrs followed by a much slower decrease to pH 4 spanning 48 hrs. In Figure 5b, we examine the corresponding change in elastic (G’) and viscous (G’’) upon GDL addition. For comparison purposes, results of a sample without any enzyme are also included. This sample was incubated at 50 °C for 5 hrs to mimic the procedure that the enzyme treated samples received. Gelation of both samples was monitored in situ in the rheometer at a constant strain amplitude (0.1%) and frequency (1 rad/s).

Several features are apparent from Figure 5b. First, there is a substantial increase in moduli with acidification as repulsion between protein chains starts to diminish with a decrease in pH. Most of this increase occurs within the first two hours consistent with the initial pH decay to 4.7 observed in Figure 5a. Second, following the initial increase in moduli, we observe a slight decrease in their value with time. The maximum in moduli may be attributed to the sample passing through the isoelectric point. Third, the elastic (as well as the viscous) modulus of the enzyme-treated sample is an order of magnitude higher than that of the sample containing no enzyme. Finally, the elastic modulus of both samples is larger than their corresponding viscous modulus, suggesting presence of a gelled network.
It is pertinent to verify the presence of this gelled network upon acidification, since the previous results were monitored at a single frequency. Figure 6 shows the frequency spectrum of the elastic and viscous modulus of both samples (circles corresponding to enzyme treated and squares without enzyme treatment) after 48 hours of acidification. For both cases, we find $G'$ and $G''$ to exhibit a very weak frequency dependence, with $G'$ larger than $G''$ over the entire frequency ranged observed. Such features are characteristics of a gel. In addition, the sample treated with enzyme has a higher elastic modulus compared to the untreated sample. Since $G'$ is related to the degree of crosslinking, this suggests presence of additional crosslinks in the enzyme-treated sample.

An important issue to consider when examining gel modulus is the extent of disulfide linkages formed upon heating the sample at 50 °C for 5 hrs and its impact on gel rheology. To examine this, samples were prepared in which N-ethylmaleimide (NEM) was added to the whey protein solution (pH 8) prior to heating at 50 °C for 5 hrs. NEM is believed to prevent disulfide bonding by blocking the free sulfhydryl groups. The samples were then cold set by GDL to pH 4. The frequency spectrum of the dynamic moduli of such a sample is shown in Figure 6 (triangles). We find both $G'$ and $G''$ of the samples with and without NEM to be essentially identical, thereby demonstrating several important points. First, the number of disulfide linkages formed during the polymerization step is few, consistent with our SDS-PAGE results (Figure 2c). Second, these disulfide linkages have negligible effect on gel rheology, with physical interactions being the primary mechanism for gel formation (without enzyme). Finally, the enhanced
modulus observed upon enzyme treatment can be directly attributed to the additional enzyme catalyzed bonds formed.

4.3.4 LARGE STRAIN AND YIELD BEHAVIOR

Figure 7a shows the dynamic moduli of the enzyme-treated and untreated samples as a function of increasing strain amplitude. At low strains, both G’ and G’’ are flat, reminiscent of materials in the linear viscoelastic regime. With increasing strain, however, nonlinearity sets in with a slow decrease of both G’ and G’’. Subsequently, G’ and G’’ tends to cross over as the material microstructure gets disrupted. Eventually at large strains, G’’ becomes larger than G’ and both decrease rapidly as the material yields and/or fracture. Qualitatively, we find the yield strain of the enzyme-treated sample to be larger than the untreated sample. However, it is difficult to pinpoint the yield (or critical strain) exactly. One approach to do this has been to draw asymptotes through the low and large strain values of G’ (as shown in Figure 7a) and call the intersection point the yield point (41;42). However, such an approach can produce errors because of the variation possible in drawing the asymptotic lines. It is interesting to note reversible “healing” of sample microstructure is possible in the immediate vicinity of the yield strain regime (data not shown) if the material is left to relax.

An alternative and precise way to determine fracture/yield strain or stress is to plot the elastic stress, defined as a product of the elastic modulus and strain, (G’γ), as a function of increasing strain (41;42;48) Such an approach has been used effectively for particulate (41;42) and polysaccharide (41;42) gels to locate the yield point, which corresponds to the maximum in the plot. Figure 7b shows the elastic stress as function of increasing strain for enzyme-treated and untreated samples. In both cases, we observe a
sharp maximum that corresponds to the yield/fracture stress and strain. In our samples, this point possibly corresponds to fracture rather than yield as samples beyond this strain showed no reversibility (data not shown). In fact, a fracture surface was visually identified in the rheometer geometry at/beyond the point of maximum stress. In addition, the strain values corresponding to the maximum stress in this figure is higher than that obtained from the yield point in Figure 7a.

We find from Figure 7b that the fracture stress of the enzyme-treated sample is about an order of magnitude higher than the untreated sample whereas the fracture strain is larger by about 50%. The same result is also obtained if we plot of the total stress (instead of the elastic stress) as a function of strain (data not shown). This is because the dominant component of the total stress is the elastic stress. Table 1 summarizes the values for the yield and fracture strains and stresses. The higher values of the yield/fracture strain and stress, as well as the elastic modulus, in the case of enzymatic treatment can be attributed to the presence of chemical crosslinks (ε-(γ-glutamyl)lysine bonds) that strengthens the network. In contrast, the absence of such chemical crosslinks in the untreated sample causes the gel network to yield/fail at lower strain and stress values.

4.3.5 COLD-SET, ENZYME-TREATED VERSUS CONVENTIONAL HEAT TREATED GELS

Protein gels are commonly prepared though heat treatment, typically at 80 °C. This produces complete heat denaturation of protein molecules leading to hydrophobic, hydrogen, and electrostatic interactions together with disulfide bond formation (49-51). Conventional gels at pH 4 were obtained by heating whey protein solution (7.5 % w/w,
pH 4) at 80 °C for specified time intervals. **Figure 8** compares the frequency spectra (at 25 °C) of both conventional heat-set gels and an enzyme treated cold-set gel. In this regard, heat-set gels obtained using two different heating times, 1 and 5 hrs, are shown. We find conventional gels obtained after 1 hr of heating to have a lower G’ than the enzyme-treated gels whereas that heated for 5 hrs exhibits a slightly higher G’ than the enzyme-treated sample. The latter can be a result of the denaturation of most of the protein chains in the conventional gels, which are strengthened by physical interactions with prolonged heating, whereas only part of the protein chains in the cold set gel denatures. The higher degree of denaturation allows more protein chains to be part of the network forming the gel, thereby leading to a larger modulus. In fact, the inset in **Figure 8** shows G’ of conventional gels (at 80 °C) as a function of heat treatment time. We find that the elastic modulus of these gels increase with heating time and asymptotes out after about 4 hours.

**Figure 9** compares the large strain behavior of the two conventional gels (1 and 5 hr heat treated) to an enzyme-treated one. In **Figure 9a**, which shows G’ for the three samples, we find nonlinearity and yielding of the conventional gels to precede that of the enzyme-treated one. This seems to hold true regardless of the heat treatment time and initial modulus of the conventional gels. **Figure 9b** plots the elastic stress as a function of increasing strain for these three samples. We not only find a substantially larger fracture strain for the enzyme treated gel but also a higher fracture stress. A comparison of the yield stress and strain values of a cold set sample without enzyme, a cold set sample with enzyme and conventional heat-treated gels is given in **Table 1**. The fracture stress of the enzyme treated sample is larger than both the untreated sample and heat-treated sample
(5 hrs) by about a factor of ten and three, respectively. The fracture strain of the enzyme-treated sample is one and a half and six times larger than the untreated sample and heat-treated sample, respectively. It is interesting to note that although the conventional gel with 5 hrs of heat treatment has a higher elastic modulus, its yield and fracture properties are much lower in value compared to the enzyme-treated gel. These results highlight the fact that the elastic modulus alone is insufficient to characterize whey protein gels without knowledge of its yield and fracture properties. Interestingly, yield properties are of special importance in food application as they mimic the chewing and biting of food.

Figure 10 shows SEM images of the microstructures of whey protein gels obtained under different conditions, conventional (5 hrs), cold set without enzyme treatment, and cold set with enzyme treatment. The conventional gel shows large voids and a non-homogenous particulate structure. These large voids in the structure may be the reason for the lower values of fracture stress and strain. This is because fracture occurs at the weakest parts of the gel network, which are expected to be the large voids regions. On the other hand, both cold set gels (with and without enzyme treatment) reveal a more homogeneous network structure. Although the cold-set gel without enzyme treatment appears more uniform and homogenous than its enzyme-treated counterpart, it has lower fracture properties. This can be attributed to the presence of additional chemical crosslinks (ε-γ-glutamyl)lysine bonds) in the enzyme-treated gel, making it capable of carrying larger stresses with higher reversible extensibility (rubberiness).

4.4 CONCLUSIONS

In this study, we examined the rheological characteristics of a low pH whey protein gel prepared using a new enzyme-catalyzed two-step process. The first step
involved enzymatic polymerization at pH 8 and 50 °C using transglutaminase. The second step entailed formation of gels at pH 4 through acidification of the polymerized whey using GDL under cold-set conditions. The alkaline conditions for the first step was chosen based on the relative activity of enzyme under different pH and its ability to polymerize β-lactoglobulin, the major component of whey protein, at this pH. Enzyme catalysis during this process led to the formation ε-(γ-glutamyl)lysine bonds and a substantial increase in molecular weight and sample viscosity. The low pH gels obtained through the second acidification step exhibited substantially higher fracture/yield stress and strain compared to cold set gels with no enzyme and conventional heat set gels at 80 °C. In this regard, we used a new approach to obtain yield/fracture properties from dynamic rheological experiments. The elastic modulus of the enzyme-catalyzed gel was also higher than the one without enzyme. Interestingly, the modulus of the heated treated sample was a function of heating time and lower than the enzyme treated sample except for samples heated for a prolonged period (~5 hrs). Even these gels had comparable or slightly higher modulus than the enzyme treated sample but considerably lower fracture stress/strain. These results taken together indicate the use of transglutaminase enzyme as a viable approach to produce low pH gels with enhanced rheological characteristics.
ACKNOWLEDGMENTS

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4.5 REFERENCES


**Figure 1.** Effect of pH on the relative activity of transglutaminase enzyme at 50 °C.
Figure 2. SDS-PAGE bands for (a,c) whey protein isolate samples, (b) α-lactoglobulin and β-lactoglobulin samples. (a) Lane 1: molecular weight markers in Daltons, lanes 2,4, and 5 are native whey protein isolate samples. Lanes 3, 5, and 7 are whey protein sample treated with enzyme at pH 6, pH 7, and pH 8, respectively. Heating for all samples was conducted at 50 °C for 5 hrs; (b) Lane 1: molecular weight markers, lanes 2,3,4, and 5 are α-lactalbumin samples, lane 2, native, lane 3,4 and 5 incubated with enzyme for 5 hrs at pH 6, 7 and 8, respectively, lanes 6,7,8, and 9 are β-lactoglobulin samples, lane 6, native, lane 7,8, and 9 incubated with enzyme for 5 hrs at pH 6, 7, and 8, respectively. (c) Lane 1: molecular weight markers, lanes 2-8 are whey protein isolate samples at pH8, lanes 3, 5, and 7 were analyzed under non reducing conditions, while lanes 2, 4, 6, and 8 were analyzed under reducing conditions. Lane 2: unheated, lanes 3 and 4: heated for 1 hr at 80 °C, lanes 5 and 6: heated for 5 hrs at 50 °C, lanes 7 and 8: incubated with enzyme for 5 hr at 50 °C.
Figure 3. SDS-PAGE bands for enzymatic polymerization of whey protein isolate samples at different (a) enzyme and (b) NaCl concentrations. All experiments were conducted at pH 8. (a) Lane 1: molecular weight marker, lanes 2 is native whey protein isolate sample. Lanes 3 to 10 are whey protein isolate samples treated with enzyme at 50 °C for 5 hrs at different concentrations as follows: lane 3 [1 U/gm proteins], lane 4 [5 U/gm proteins], lane 5 [10 U/gm proteins], lane 6 [20 U/gm proteins], lane 7 [30 U/gm proteins], lane 8 [40 U/gm proteins], lane 9 [50 U/gm proteins], and lane 10 [100 U/gm proteins]; (b) Lane 1: molecular weight marker, lanes 2 is native whey protein isolate sample. Lane 3 is sample heated at 50 °C for 5 hrs without enzyme. Lanes 4 to 8 are samples treated with 10 U/gm of enzyme at 50 C for 5 hrs. Lane 4 [no salt added], lane 5 [20 mM NaCl], lane 6 [50 mM NaCl], lane 7 [100 mM NaCl], lane 8 [200 mM NaCl].
Figure 4. Apparent viscosity (at a shear rate of 50 s$^{-1}$) of whey protein isolate samples as a function of incubation time with enzyme (100u/g) at 50 °C. Results are shown for different pHs.
Figure 5. Changes in whey protein properties during cold-set acidification through addition of 1.85% glucono-δ-lactone (GDL) acid. The original sample was enzyme treated at pH 8. (a) Change in pH with time upon addition of GDL. Inset at the upper right corner is an enlarged view of the initial pH decrease with time; (b) evolution of the elastic ($G'$) and viscous ($G''$) moduli of the enzyme-treated whey protein sample during decrease of its pH.
Figure 6. Dynamic frequency spectrum of the elastic ($G'$) and viscous ($G''$) moduli of acidic (pH 4) cold-set gels. Results are compared for a sample that has been enzyme treated prior to acidification with samples without enzyme pretreatment. Circles represent sample with enzyme treatment. Squares represent sample incubated at 50 °C for 5 hrs without enzyme. Triangles represent sample treated with NEM (to prevent disulfide linkages) and incubated at 50 °C for 5 hrs without enzyme (hollow triangles: $G'$, filled triangles: $G''$)
Figure 7. Effects of increasing strain amplitude on the (a) dynamic moduli and (b) elastic stress (product of elastic modulus and strain) of cold-set, acidic gels. The intersection of the asymptotes in (a) corresponds to the “yield” point. All experiments were conducted at a frequency of 1 rad/sec.
Figure 8. Comparison of the frequency spectrum of the dynamic moduli of an acidic cold-set gel (enzyme pretreated) with conventional gels, all measured at 25 °C. The conventional gels were prepared at the same pH (of 4) of the acidic, cold-set gel through heat treatment at 80 °C for different time intervals. The inset in the upper left corner shows the evolution of $G'$ and $G''$ (at 80 °C, $\omega=1$rad/s) as a function of heating time for the conventional gel.
Figure 9. Comparison of the large strain behavior of a cold-set acidic gel (enzyme pretreated) with conventional, heat-treated gels at the same pH (4). (a) elastic modulus ($G'$) as a function of strain amplitude; (b) elastic stress ($G'$) showing maximum with increasing strain amplitude.
Figure 10. SEM micrographs of acidic gels (pH 4) obtained under different conditions: (a) conventional, (b) cold set without enzyme, (c) cold set with enzyme. All samples are shown at a magnification of 10,000x. Bars correspond to 1 µm.
Table 1. Yield and fracture strains and stresses of acidic gels.

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CHAPTER 5
MODIFICATION OF ACIDIC WHEY PROTEIN GELS BY CROSSLINKING WITH TRANSGlutaminase ENZYMEnE

Chapter 5 is essentially a manuscript by Ahmed Eissa and Saad Khan that has been submitted to the Journal of Agricultural and Food Chemistry in December 2004
Modification Of acidic Whey Protein Gels By Crosslinking With Transglutaminase Enzyme

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ABSTRACT

Low pH whey protein gels are formulated using a sequential protocol of heat treatment, enzyme incubation and cold set acidification. The heat-induced disulfide and enzyme-catalyzed ε-(γ-glutamyl)lysine linkages, both at neutral pH, produce a polymerized protein solution. The molecular weights of these samples show an exponential increase with protein concentration. The additional enzyme-catalyzed crosslinks cause little change in molecular weight from that of heat-treated samples at low protein concentrations, indicating predominant intramolecular crosslinking. Enzyme treatment at higher protein concentration however causes increase in molecular weight possibly due to formation of intermolecular crosslinks. Acidification of the polymerized protein solutions through glucono-δ-lactone acid leads to gel formation at pH 4. The elastic (G’) and viscous (G’’) moduli of gels with and without enzyme treatment show similar frequency dependence indicating similar microstructures, consistent with all samples exhibiting similar fractal dimensions of ~2 obtained independently using rheology and confocal microscopy. A substantial increase in fracture strain and stress of the gel was achieved by the enzyme treatment. However, elastic modulus (G’) is slightly larger after enzyme treatment compared with heat-treated samples. These results indicate that factors responsible for fracture properties may not be apparent in the gel microstructure and linear viscoelastic properties.

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5.1 INTRODUCTION

Whey proteins have become an important ingredient for many food products because of their high nutritional value, easy digestibility and capacity to impart functional characteristics that include emulsification, stabilization, foaming and gelation (1-3). The ability of whey proteins to form gels is particularly desirable as gelation plays an essential part in dictating the texture of the final food products (4-7). Typically gelation, which involves formation of a three-dimensional polymeric network, is achieved through heat treatment of whey proteins at high or moderate ionic strengths, and/or close to the isoelectric point (5;7-12). These gels are often referred to heat-set gels as the single heat treatment step leads to gel formation through protein denaturation and polymerization (disulfide crosslinks) and subsequent physical bond formation. Gels can also be produced using a two step process that involves heat treatment at low ionic strength and/or far from the isoelectric point, followed by increase in ionic strength and/or adjustment in pH (13-15). These gels are labeled as cold-set gels as the initial heat treatment produces a polymerized solution with gelation occurring during the subsequent cold set conditions through screening of the repulsive forces. Such an approach is commensurate with some food applications, in which it is not desirable to heat the products to high temperatures to induce gelation, and it becomes advantageous to induce gelation at ambient or near ambient temperatures (16).

An area of importance in which whey protein gels are having limited use is in low pH food products. This is because heat-set whey protein gels under acidic conditions (pH < 4.6) exhibit poor rheological properties such as brittleness and weakness due to the reduction of the strong disulfide bonds at such acidic conditions and pH-associated
effects on the denaturation and aggregation reactions (17). The rheological properties of such gels can however be improved by introducing additional intermolecular chemical crosslinks. A viable approach in this regard is to use enzymes (18-22). For instance, transglutaminase (TG, EC2.3.2.13) can be used to crosslink whey proteins (22-29) through crosslinking glutamine and lysine residues as it catalyzes the acyl transfer reaction and produces $\varepsilon$-(\$\gamma$-glutamyl)lysine bonds.

Previous and current work in our laboratory has focused on enzyme-facilitated gelation of whey proteins at low pH (25). In our first approach, we developed cold-set whey protein gels at low pH with enhanced rheological properties by crosslinking the proteins with transglutaminase enzyme under alkaline conditions. The alkaline conditions were chosen to partially denature the protein and enable crosslinking by enzyme. However, the enzyme activity decays more rapidly under alkaline conditions than at neutral pH. In addition, the maximum temperature used in the process (50 °C) may not have allowed formation of all possible disulfide linkages.

To circumvent these issues as well as introduce new functionality, we examine in this study a different approach for modifying whey protein gels. In this protocol as illustrated in **Figure 1**, whey protein solution at pH 7.0 is preheated at 80 °C for 1 hr, followed by enzyme treatment at 50 °C. The ensuing polymerized solution is gelled through slow (room-temperature) acidification to pH 4 by adding glucono-\(\delta\)-lactone (GDL) acid. The distinguishing features of this approach are the use of neutral pH and the preheating step to eliminate the drawbacks of our prior work. The disulfide linkages formed during the preheating step also enables us to (i) tailor gels with both disulfide and enzyme-catalyzed bonds and (ii) understand the added functionality of the $\varepsilon$-(\$\gamma$-
glutamyl)lysine bonds in cold set gels in reference to the traditional cold set gels containing only disulfide linkages. As such, we examine in detail the gelation process and gel properties for formulations with and without enzyme. Linear and non-linear rheological experiments are conducted to characterize and understand gel behavior. This is supplemented with confocal laser scanning microscopy (CSLM) to directly probe gel microstructure and obtain fractal dimensions that can be compared with that acquired through rheology.

5.2 MATERIALS AND METHODS

5.2.1 MATERIALS

Whey protein (WP) isolate was obtained from Davisco Food International (LeSueur, Mn) and used as received. A commercial version of transglutaminase enzyme (1% enzyme and 99% maltodextrin, by weight) was provided by Ajinomoto Company (Japan). Sodium azide, glucono-δ-lactone (GDL), N-ethylmaleimide (NEM), sodium dodecyl sulfate (SDS) and urea were obtained from Sigma Chemical Co. (St.Louis, MO). Deionized water (>15MΩ) was used in all the experiments.

5.2.2 PREPARATION OF PROTEIN SOLUTIONS

The stock protein solution was prepared by dissolving WP powder in deionized water to the desired final concentration at pH 7 and stirring for 1 hr to ensure complete solubility. The resulting solution was deaerated for 30 minutes under vacuum of 30 inch Hg at room temperature to eliminate trapped air bubbles.

Polymerization of the stock solution was carried out by preheating the solution at 80 °C for 1 hr to denature the protein and induce disulfide bonds. In the case of samples treated with enzyme, enzyme was added at 10 units/gm of protein after the preheating
step. The samples were then stirred for about 20 minutes and incubated at 50 °C for 10 hrs. Each unit of enzyme is defined in terms of its activity, which corresponds to the amount of the enzyme necessary to catalyze the reaction of benzylloxycarbonyl-L-glutaminylglycine and hydroxyl amine to yield 1µmole of hydroxamic acid per minute at 37 °C.

5.2.3 PREPARATION OF GELS

WP gels at pH 4 were obtained by adding GDL to the heat- or heat+enzyme-treated WP solutions (0.2 gm GDL per gm of protein) at room temperature and stirring for 15 minutes. For in situ measurements of gelation and subsequent gel properties, the solutions were then poured into a rheometer cup for rheological measurements.

5.2.4 GEL ELECTROPHORESIS

Polyacrylamide gel electrophoresis (PAGE) was performed using a Mini-PROTEAN 3 Electrophoresis Cell (BIO-RAD, Hercules, CA). Protein solutions (5%) were mixed with 40 volumes of sample buffer (40% glycerol, 8% SDS, and the balance being deionized water). β-mercaptoethanol was added (50 µL per 950 µL of sample buffer) when reducing conditions were needed. The mixture was heated for 5 min at 95 °C and then subjected to electrophoresis in 5-15% polyacrylamide gels using the discontinuous system. These gels were then stained with 0.1% Coomassie blue and then destained by deionized water for 2 hrs. The gels were subsequently immersed in drying solution (containing 20% ethanol and 10% glycerol in deionized water) for 30 minutes and fixed in drying frames for 24 hrs. The dry gels were finally scanned and the bands analyzed in terms of molecular weights.
5.2.5 RHEOLOGICAL MEASUREMENTS

Dynamic rheological measurements were conducted on an ARES rheometer (TA Instruments, New Castle, DE) at 25 °C, using a couette geometry. In these experiments, the samples were subjected to a sinusoidal deformation as either a function of increasing strain amplitude or frequency of oscillation, and the corresponding elastic (G’) and viscous (G’’) moduli were measured. The strain sweep experiments, which were conducted at a constant frequency of 1 rad/s, served two purposes. First, it provided the limit of linear viscoelasticity (LVE) that could be used in the frequency experiments. Second, as discussed later, it provided a method to examine yield and fracture stress/strain of the samples (30;31). The frequency spectrum of the elastic and viscous modulus, on the other hand, provided a signature of the state (e.g., liquid or gel) of the samples (32). In all experiments conducted, the samples were covered with n-hexadecane to prevent evaporation.

5.2.6 SIZE EXCLUSION CHROMATOGRAPHY (SEC)

A Waters 2690 Separation Module (Waters, Milford, MA) size exclusion chromatography (SEC) unit attached to a DAWN-DSP Laser Photometer (Wyatt Technology, Santa Barbara, CA) multi-angle laser light scattering (MALLS) instrument was used for analyzing molecular weights and aggregate sizes of the protein samples. A series of 3 columns (Waters Ultrahydrogel 120, 500 and 2000) was used to separate molecular weights in the range of (10 kDa to 7,000 kDa). Interferometric refractometer (Optilab DSP) and ultra violet detector (Waters 966 Photodiode Array Detector) were used as concentration detectors. The eluting solvent consisted of 10 mM Tris buffer (pH 7), 100 mM NaCl and 0.02% sodium azide. The value of dn/dc (where n is refractive
index, and c is the protein concentration) was taken as 0.19 ml/mg (33). Flow rate through the SEC was maintained at 0.5 ml/min in all the runs.

5.2.7 CONFOCAL MICROSCOPY

A confocal laser scanning system (Leica TCS SP) attached to an inverted microscope (Leica DM IBRE, Leica Microsystems, Wetzlar, Germany) was used to obtain confocal images of the gel microstructure. Samples dyed with Rhodamine B (0.01%) were viewed with a 100x numerical aperture 1.4 oil immersion objective. A spacer of 0.12 mm thickness was adhered on the microscope slide. Immediately after acidulation with GDL, 25 µL of protein solution was poured onto a microscope slide fitted with 0.12 mm spacer. The sample was then covered with a glass cover and viewed after 24 hrs of acidification.

5.3 RESULTS AND DISCUSSIONS

5.3.1 POLYMERIZATION OF WHEY PROTEINS BY DISULFIDE INTERCHANGE AND ENZYMATIC CROSSLINKING

Previous work (25) in our laboratory revealed that whey proteins (WP) cannot be crosslinked by transglutaminase enzyme in the native state at pH 7. However, heating the WP to a temperature higher than the denaturation temperature enables enzyme-catalyzed crosslinking to occur. This is illustrated in the SDS-PAGE analysis of Figure 2, which shows the effects of heat treatment and enzyme incubation on whey protein molecular weight. Three samples are portrayed in the figure, a native WP sample, a WP sample heated to 80 °C for 1 hr (lanes 3 and 5), and a WP sample heated to 80 °C for 1 hr and then incubated with transglutaminase at 50 °C for 10 hrs (lanes 4 and 6). Under non-reducing conditions, i.e., without the presence of β-mercaptoethanol (lanes 3 and 4), high
molecular weight aggregates are noticed on the top of the gel, with and without enzyme. However, under reducing conditions (lanes 5 and 6), we observe only monomeric bands in the absence of the enzyme (lane 5), corresponding mainly to β-lactoglobulin and α-lactalbumin. The disulfide bonds created in the preheating step have been cleaved by the β-mercaptoethanol used in the electrophoresis run. In the presence of enzyme (lane 6), on the other hand, we clearly notice presence of high molecular weight aggregates at the top of the gel. These aggregates can be attributed to polymerization through enzyme-catalyzed ε-(γ-glutamyl)lysine bonds since the disulfide bonds have been cleaved by the reducing agent.

To investigate in more details the molecular weight distribution, samples were examined using size exclusion chromatography. Figure 3 shows chromatograms of native whey proteins and proteins subjected to heat or combined heat and enzyme treatments. The native whey proteins exhibits three peaks 1, 2 and 3 corresponding to bovine serum albumin (BSA), β-lactoglobulin(β-lg) and α-lactalbumin (α-lac), respectively. Preheating the protein at 80 °C for 1 hr produces higher molecular weight aggregates (polymer) from disulfide interchange polymerization (34-47), as seen in the peaks eluting earlier than the native peaks. Upon treatment with enzyme, we expect an increase in molecular weight due to formation of additional crosslinks. However, the chromatograms in Figure 3 show that the effect of enzyme is highly dependent on whey protein concentration. At 3% (curve b), the large aggregate peak (eluting at ~ 43 min) is not affected by enzyme treatment, whereas at 7.5% (curve c), the large aggregate peak moves to earlier elution time with enzyme treatment. We also observe another peak eluting at ~ 51 min for 3% and 7% protein concentration prior to enzyme treatment. This
peak were found to have an average degree of polymerization of 3 (based on a β-lg monomer of 18,600 Da). After enzyme treatment, this peak moves to a slightly lower elution time (~49 min) and the average degree of polymerization increases to 5, suggesting formation of intermolecular crosslinks. It is not clear, however, why these oligomers are undergoing intermolecular crosslinking by the enzyme while the larger aggregate at 3% is not. Further investigation is needed to explain this phenomenon.

We observe in Figure 3 that heat treatment of a 7.5% WP solution results in the appearance of a small shoulder to the left of the main aggregate peak, indicating onset of a bimodal distribution. At the same protein concentration but with additional enzyme treatment, we find the bimodal distribution of molecular weight to become more evident with the presence of separate peaks. The bimodality at higher WP concentrations may be attributed to a retarded termination rate in what is known as Trommsdorff effect (48). In this case, an increase in solution viscosity causes auto acceleration of polymerization (48), slowing down diffusion of the growing polymers, and causing a drop in the termination rate. The Trommsdorff effect is expected to take place mainly in the disulfide interchange polymerization, which is a typical chain growth polymerization. Enzymatic crosslinking takes place after the disulfide interchange polymerization and causes further separation of the two aggregate peaks. For clarity throughout the paper we will use the terms “primary aggregates” to account for the protein chains that are polymerized via disulfide bonding and “secondary aggregates” to indicate primary aggregates that are further crosslinked by transglutaminase.
5.3.2 EFFECT OF CONCENTRATION ON MOLECULAR WEIGHT

Figure 4a shows the weight average molecular weight of the protein aggregates as a function of protein concentration. At low protein concentrations (<5%), the weight average molecular weight shows very little change after enzyme treatment; however, at higher concentrations, enzyme treatment increases molecular weight considerably. The fact that enzyme crosslinking does not increase the molecular weight at low concentrations can be explained as follows. At low protein concentrations, especially in a repulsive environment, the enzyme tends to produce intramolecular crosslinks of the primary aggregates. At higher WP concentrations, the primary aggregates tend to overlap extensively and increase the chance of intermolecular crosslinking.

Figure 4b shows the polydispersity index (PDI) of the primary and secondary aggregates. PDI falls between 1 and 1.5 at low protein concentrations, as expected in a typical chain growth polymerization. At higher concentrations, ~ 5% and higher, the PDI exceeds 1.5 due to the Trommsdorff effect mentioned earlier. Subsequent polymerization by enzyme crosslinking increases the PDI further, with the effect being more pronounced at higher protein concentrations.

5.3.3 RHEOLOGICAL CHARACTERIZATION OF COLD SET GELATION BY GDL

Primary as well as secondary aggregates were slowly acidified by adding GDL and allowing them to set at 25 °C. The amount of GDL needed was found to be linearly dependent on protein concentration (0.2 g GDL per gm of protein). Figure 5 shows the evolution of the elastic (G′) modulus with time for samples with and without enzyme treatment. From the inset in the figure, which shows the evolution of both the elastic (G′)
and viscous ($G''$) moduli during the initial time period, we observe $G''$ to be larger than $G'$ immediately after GDL addition. This is consistent with the fact that the sample following heat and enzyme treatment is a solution and not a gel. However, both moduli increase rapidly with time with $G'$ crossing over $G''$ as elasticity starts to dominate. We notice a maximum in $G'$ for all samples shown in Figure 5 at ~2 hrs corresponding to the isoelectric point of the protein at pH ~5 (25;26). While this feature is common to both heat and combined heat and enzyme treated samples, several distinctive differences can be observed between these two types of samples. First, the elastic modulus is modestly higher for samples with enzyme treatment. Second, the reduction in $G'$ with time (after reaching its maximum) is smaller for samples with enzyme treatment than their counterparts with only heat treatment. Finally, this lower sensitivity of $G'$ to pH reduction or time is more pronounced for samples with higher WP concentrations. The last two features are observed clearly when one compares the value of $G'$ after 24 hrs with respect to the maximum ($G'/G'_{\text{max}}$) as a percentage (Table 1). We find that $G'$ after 24 hrs (at pH 4) is less than 50% of its maximum value when the sample has been heat treated only. In contrast, gels prepared with additional enzyme treatment retain 78 and 89% of their maximum $G'$ values for WP concentrations of 5 and 7% respectively. The reasons behind these observations may be explained as follows. As the pH decreases below the isoelectric point, the chains start to repel each other electrostatically because of the net positive charge on the molecules. Consequently, the network, which is held together through physical bonds, is weakened as some bonds dissociate. This leads to a decrease in $G'$. The use of enzyme produces additional crosslinks that keep the three
dimensional network more stable against electrostatic repulsion. The effect is more pronounced at higher protein concentration as there are more enzyme crosslinks.

### 5.3.3.1 GEL RHEOLOGY

**Figure 6** shows the frequency spectrum of $G'$ and $G''$ for samples at pH 4. We find both heat and heat+enzyme treated samples to exhibit gel-like features with $G'>G''$ and both moduli exhibiting very weak frequency dependence. While the enzyme-treated gels exhibit slightly larger $G'$, it is interesting to note that the mechanical spectra of both gels look very similar with $G'$ scaling as $\sim \omega^{0.1}$.

**Figure 7** shows the effect of whey protein concentration (or volume fraction) on the elastic modulus of gels. We find $G'$ of the enzyme-treated samples to be consistently higher than the samples without enzyme treatment. In both cases though, $G'$ exhibits a power-law behavior with protein volume fraction $\phi$, $G' \sim \phi^n$, with the power-law exponent $n$ varying between 4.4 and 4.6 for the two types of gels. The power-law dependence of $G'$ together with the similar values for $n$ observed for both types of gels suggest that the gels are fractal in nature and that they possess similar microstructures (49-51). We examine these issues in more detail in the next section.

The large strain behavior of the enzyme-treated gel is, however, substantially different than the gel which has been exposed to heat treatment only. This is illustrated in **Figure 8**, which compares the elastic stress (product of shear stress and strain) as a function of increasing strain of the two types of gels (25;30;31). The maximum in the plots correspond to the fracture of the gel and has been visually observed as well. We find the gel treated with enzyme to fracture at higher stress and strain values. In fact, the fracture strain is about an order of magnitude higher while the fracture stress is three-fold
larger. The higher fracture strain value (i.e., more deformability of the gel) may be due to the additional ε-(γ-glutamyl)lysine bonds created by the enzyme. These additional crosslinks not only affect the fracture values but also alter the shape of the elastic stress curve. In the case of the gel without enzyme treatment, we observe only two regions: an initial linear regime up to the maximum and then a very steep decrease in stress following the linear regime. In contrast, gels with enzyme-catalyzed ε-(γ-glutamyl)lysine bonds reveal three regimes: an initial linear regime, an intermediate non-linear regime leading to the elastic stress maximum and gradual decrease in elastic stress following the maximum.

5.3.3.2 FRACTAL DIMENSION AND MICROSTRUCTURE OF GELS

We examine first the fractal dimensions of the gels using a rheological approach suggested by Shih and coworkers (52). According to these authors, the first step towards doing this is to determine whether a gel is strong-linked or weak-linked. In strong-linked gels, the links between flocs are stronger than the links within the flocs. As a result, failure under deformation occurs through breaking of the intra-floc linkages. This manifests itself as a decrease in the linear viscoelastic region with increasing sample concentration. The limit of linearity is defined in the literature as the value of the strain at which there is an appreciable deviation in the elastic modulus from the linear viscoelastic plateau value. This value ranges from 5% to 30% (52;53). In the case of weak-linked gels, the intra-floc bonds are more rigid than the inter-floc links, and failure occurs in the inter-floc links. In this case, the limit of linearity increases with concentration. To evaluate the category of our gels, we plot in Figure 9 the elastic modulus (G’) as a function of strain for different protein concentrations. We notice quite clearly that the
onset of non-linearity (regardless of how one defines it) shifts to lower values with increasing concentration, indicating our gels to be strong-linked. The same trend was observed for the gels with enzyme treatment (data not shown).

In a strong linked gel, $G'$ scales as $G' \sim \phi^{(d+x)/(d-D)}$, where $x$ is the backbone fractal dimension of the flocs, $d$ is the Euclidean dimension, and $D$ is the gel fractal dimension. The backbone fractal dimension ranges from 1 to 1.3 for colloidal gels (54).

From an experimental standpoint, we find from Figure 7 that $G' \sim \phi^n$ for our gels with $n$ corresponding to 4.4±0.4 and 4.6±0.3 for samples with and without enzyme treatment respectively. Equating the theoretical and experimental power-law exponents, and substituting $x = 1.15$ and $d = 3$, we find $D \approx 2.1$ for both types of gels. The same value of the fractal dimension suggests very similar structures for both types of gels. It is important to emphasize that the arbitrary chosen value of $x = 1.15$ has a negligible effect on the final value of $D$. A difference of 0.3 in the value of $x$ (which accounts for the maximum change of $x$ from 1 to 1.3) changes the value of $D$ by only ~2%. The value of the fractal dimension approximating 2.0 suggests Reaction Limited Aggregation (RLA) (52). Such mechanism is expected for the slow aggregation processes. This agrees with the slow acidulation in our case with GDL. An interesting point to note in Figure 9 is the presence of strain hardening behavior prior to failure/fracture. Such behavior has been observed in the literature (55) and is attributed to simultaneous creation and loss of junctions between molecules, with the rate of creation being slightly higher than the loss. We refrain from a detailed discussion of the strain hardening behavior of our system as this is beyond the scope of this work.
An alternative approach for determining the fractal dimension, as well as directly visualizing microstructure, is through use of confocal microscopy (56). Figure 10 shows confocal images of the heat- and heat+enzyme- treated gels for two different WP concentrations. We observe all gels to exhibit fine stranded structures. More importantly, we find the microstructures of the gels to be independent of the preparation method. The fractal dimensions of the gels were calculated from the images by the box counting technique using Image J software. In this technique, the fractal dimension can be obtained from the scaling relation \( N \propto r^{-D} \), where \( N \) is the number of boxes filled with protein flocs, \( r \), the size of the square box and \( D \), the fractal dimension of the protein network. Table 2 provides values of \( D \) obtained using both confocal microscopy and rheology. We find the fractal dimension of all gels obtained via confocal microscopy, regardless of treatment type or concentration, to have the same value of ~2.0. This is in excellent agreement with that obtained through rheology.

It is interesting to note that although the microstructure as well as fractal properties of both types of gels are similar, the fracture stress and strain are considerably different for them. A similar trend has been reported by Errington and Foegeding (17) who found WP gels prepared under different conditions to exhibit similar microstructure and close values of modulus, but different fracture properties. The observation from our gels suggests that factors responsible for fracture properties may not be apparent in the microstructure, at least not in the length scales we are probing through confocal microscopy.
5.4 CONCLUSIONS

In this study, we examined the role of transglutaminase enzyme in modulating the properties of low pH (~4) whey protein gels. A multistep approach involving preheat at neutral pH, subsequent enzyme treatment at 50 °C and final cold set gelation through acidulation with glucono-δ-lactone was adopted. The disulfide and ε-(γ-glutamyl)lysine bonds formed through heat and enzyme treatment respectively produced polymerized whey protein solutions. The molecular weight of these samples increased with protein concentration with the role of additional enzyme crosslinking manifesting as higher molecular weights only at higher protein concentration. This seemed to suggest that enzyme crosslinking was predominantly intramolecular at lower protein concentrations.

During acidulation, *vis a vis*, the gelation process, the elastic modulus of the samples increased by several orders of magnitude and exhibited a maximum at the isoelectric point. The reduction in modulus as pH fell below the isoelectric point could be attributed to the development of electrostatic repulsion between the chains. This caused the network held together by physical bonds to weaken as some bonds dissociated. The use of enzymes produced additional crosslinks that retarded the modulus decrease and made the network more stable against electrostatic repulsion.

The elastic modulus of the final gels showed a modest increase for samples treated with enzyme. In contrast, the fracture strain and stress revealed significant increase with enzyme treatment. Interestingly, the microstructures of all gels, with and without enzyme treatment, were found to be similar with a fractal dimension of ~2. This common value was obtained independently using rheology and confocal microscopy.
These results taken together suggest that the nonlinear fracture/yield properties are not reflected in the microstructure of the gels, at least not in the length scales probed using confocal microscopy.
ACKNOWLEDGMENTS

The authors acknowledge the financial support of Southeast Dairy Foods Research Center. Thanks are also due to Professor Nina Allen and Dr. Eva Johannes (NCSU, Cellular and Molecular Imaging Facility) for helping with confocal imaging.
5.5 REFERENCES


[35] Wada, R.; Kitabatake, N. \( \beta \)-Lactoglobulin A with N-ethylmaleimide-modified sulfhydryl residue, polymerized through intermolecular disulfide bridge on


Figure 1. Schematic diagram for low pH whey protein (WP) gel preparation combining preheat, enzymatic treatment and cold set gelation.
Figure 2. SDS-PAGE analysis for WP isolate samples. Lane 1 gives molecular weight markers (corresponding values shown in Daltons). Lane 2 corresponds to native WP isolate sample. Lanes 3 and 5 are WP samples (pH 7) preheated at 80 °C for 1 hr (lane 3 is under non reducing conditions whereas lane 5 is under reducing conditions). Lanes 4 and 6 are WP samples (pH 7) preheated at 80 °C for 1 hr and then incubated with transglutaminase for 10 hrs at 50 °C (lane 4 is under non reducing conditions whereas lane 6 is under reducing conditions).
Figure 3. Chromatograms of WP samples subjected to different treatments: (a) native WP; (b) 3% WP sample preheated at 80 °C, with and without enzyme treatment; (c) 7.5% WP sample preheated at 80 °C, with and without enzyme treatment.
Figure 4. Weight average molecular weight (Mw) (a) and polydispersity index (PDI) (b) of primary and secondary aggregates at different protein concentration. Primary aggregates correspond to WP molecules polymerized via disulfide interchange, whereas secondary aggregates refer to primary aggregates that were further polymerized by transglutaminase enzyme.
Figure 5. Evolution of the elastic (G') modulus of WP samples upon the addition of GDL (0.2 gm per gm of protein) at 25 °C. All samples were preheated at 80 °C for 1 hr and some samples were incubated with enzyme following heat treatment (as noted in figure) prior to GDL addition. Inset reveals evolution of the elastic (G’) and viscous (G’’) moduli in the first hour for a 7% WP sample without enzyme. Squares and circles represent 7 and 5% by wt samples, respectively.
Figure 6. Elastic and viscous moduli of WP gels (pH 4) as a function of frequency, measured at 25 °C. Squares and circles represent 7% and 5% by wt samples, respectively. Viscous moduli are shown only for 5% samples. All samples were preheated at 80 °C for 1 hr.
Protein volume fraction $\phi$ (%)

$G' = 13.14 \phi^{4.4\pm0.4}$
$R^2 = 0.998$

$G' = 6.74 \phi^{4.6\pm0.3}$
$R^2 = 0.972$

Figure 7. Elastic modulus of WP gels (pH 4) as a function of protein volume fraction. Data for samples with and without enzyme treatment are shown.
Figure 8. Elastic stress of 5% WP gels (pH 4), with and without enzyme treatment, as a function of strain, measured at 25 °C and 1 rad/sec. Both samples were preheated to 80 °C for 1 hr.
Fig 9. Elastic modulus of WP gels (pH 4) at different concentrations obtained without enzyme treatment, as a function of strain. The limit of linearity shifts to lower strain values as concentration increases.
Figure 10. Confocal images of WP gels (pH 4). A and B correspond to 5% WP samples without and with enzyme treatment, respectively. C and D represent 7% WP samples obtained without and with enzyme treatment, respectively.
**Table 1.** Values of $G'_{24\text{hrs}}/G'_{\text{Max}}$ (%) of WP gels (pH 4) at 5%, and 7%, with and without enzyme treatment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Heat + Enzyme</th>
<th>Heat only</th>
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<tr>
<td>Whey protein concentration (%)</td>
<td>5</td>
<td>7</td>
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<tr>
<td></td>
<td>5</td>
<td>7</td>
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<tr>
<td>$G'<em>{24\text{hrs}}/G'</em>{\text{Max}}$ (%)</td>
<td>78.3</td>
<td>88.6</td>
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<td></td>
<td>47.8</td>
<td>49.8</td>
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**Table 2.** Fractal dimensions of WP gels (pH 4) at 5%, and 7%, with and without enzyme treatment, as obtained from rheology and confocal microscopy.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Heat + Enzyme</th>
<th>Heat only</th>
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<tbody>
<tr>
<td>Whey protein concentration (%)</td>
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<td>7</td>
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<tr>
<td>Fractal dimension from confocal microscopy</td>
<td>1.96 ± 0.02</td>
<td>1.98 ± 0.03</td>
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<tr>
<td>Fractal dimension from rheology</td>
<td>1.96 ± 0.03</td>
<td>1.98 ± 0.02</td>
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<tr>
<td>Fractal dimension from confocal microscopy</td>
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CHAPTER 6

CHEMICAL AND PHYSICAL INTERACTIONS IN
ENZYMATICALLY MODIFIED COLD-SET ACIDIC WHEY
PROTEIN GELS

A revised version of chapter 6 by Ahmed S. Eissa and Saad A. Khan will be submitted to

the Journal of Agricultural and Food Chemistry.
Chemical and Physical Interactions in Enzymatically Modified Cold-Set Acidic Whey Protein Gels

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ABSTRACT

In this study, we investigate the relative roles of physical and chemical interactions that affect the properties of protein polymers and cold-set gels at pH 4, prepared using the same protocol as in the second part of the thesis. We examine the role of hydrogen bonding, hydrophobic interactions, disulfide bonds and \( \varepsilon-(\gamma\text{-glutamyl})\)lysine bonds. Physical interactions do not play a major role in the molecular weight or the size of the polymer, prior to acidification. However, they affect the gel rheological properties profoundly. The disruption of the hydrogen bonds inhibits gelation, while the disruption of the hydrophobic interactions causes extensive syneresis and result in weak and fragile gels. Chemical bonding affects the gel elastic modulus mildly and plays a detrimental role in the fracture/yield properties. The introduction of \( \varepsilon-(\gamma\text{-glutamyl})\)lysine bonds increases the fracture strain and enhances the rheological properties of the gels that lack the disulfide bonds.

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6.1 INTRODUCTION

Whey proteins possess functional characteristics that promote emulsification, gelation, solubility, whipping/foaming and thickening (1-3) in numerous food products including dairy, confectionary, salad dressings, soups, cakes, breads, infant formulas, sausages, and desserts. Whey protein gels in particular provide water-holding matrix and textural properties that are necessary for pleasant mouth feel. The structure of these gels involves both chemical and physical interactions. Chemical interactions entail disulphide and enzymatic-catalyzed ε-(γ-glutamyl)lysine bonds, whereas physical associations occur through hydrogen bonding, and, hydrophobic and electrostatic interactions. The extent of each type of interactions determines the final rheological properties of the gels. Chemical bonding are strong (200-400 kJ/mol) and permanent, while the physical interactions are transient and weaker (5-10 kJ/mol for hydrophobic interactions, 10 - 40 kJ/mol for hydrogen bonding, and 25-80 kJ/mol for electrostatic interactions) (4).

Low pH (pH ≤ 4) heat-set whey protein gels suffer from detrimental brittleness and loss of deformability due to the absence of the covalent bonds (5,6). Remedies for such problems involve the introduction of covalent bonds to fortify the network. Disulfide bonds as well as enzyme catalyzed crosslinks have been introduced to the protein chains prior to acidification (7-11) as a means of texture enhancement. In this case, gelation is done by cold setting, where the polymerization/aggregation takes place in solution in a repulsive environment, followed by acidification, to screen the repulsion between the chains and allow gelation (9,10). In spite of several studies on cold-set whey protein gelation (12-16), the role of each type of bonds or interactions in the rheological properties have not been fully understood. In this study, we investigate cold-set whey
protein gels at pH 4. Two types of chemical bonds are introduced: disulfide (introduced via thermal treatment) and ε-(γ-glutamyl)lysine bonds (introduced via enzymatic treatment). The ε-(γ-glutamyl)lysine bonds are created using transglutaminase (EC2.3.2.13) enzyme. Three types of physical interactions are also believed to participate in the gel network structure.

1. Hydrophobic interactions take place due to the exposure of hydrophobic residues to the water interface upon denaturation. This drives the hydrophobic residues to aggregate, to minimize the interface with water.

2. Hydrogen bonds in proteins occur between carbonyl oxygen and amide hydrogen because of the slight positive charge on the hydrogen atom and the slight negative charge on the oxygen or nitrogen atoms. Hydrogen bonds occur between two adjacent hydrophilic or polar groups in proteins or between water and protein molecules.

3. Electrostatic interactions emerge from the charges on the protein surfaces. At low ionic strength, the similar charges repulse each other and tend to extend the molecules in the solvent. At high ionic strength, the molecule chains tend to collapse and aggregate.

In prior research in our laboratory, we developed whey protein gels at low pH with modified rheological properties by crosslinking with transglutaminase enzyme under alkaline (10) and neutral conditions (11). In this study, we focus on understanding the individual role of hydrophobic interactions, hydrogen bonding, disulfide and ε-(γ-glutamyl)lysine bonds in the polymerization of the protein molecules and formation of cold-set gel network at pH 4.
6.2 MATERIALS AND METHODS

6.2.1 MATERIALS

Whey protein isolate (WPI) were obtained from Davisco Food International, (LeSueur, Mn). A commercial version of transglutaminase enzyme (1% enzyme and 99% maltodextrin, by weight) was supplied by Ajinomoto Co., Japan. Sodium azide, glucono-δ-lactone (GDL), dithiothreitol (DTT), sodium dodecyl sulfate (SDS), and urea were obtained from Sigma Chemical Co. (St.Louis, MO). De-ionized water (>15MΩ) was used in all the experiments.

6.2.2 PREPARATION OF PROTEIN SOLUTIONS AND GELS

WPI powder was dissolved in de-ionized water (pH 7) to the desired concentration and stirred for 1 hr to ensure complete solubility. All the samples were preheated at 80 °C for 1 hr to denature the protein and induce disulphide bonds. For enzymatic treatment, the enzyme was added after the preheating step and stirred for 20 minutes and incubated at 50 °C for 12 hrs. WPI gels at pH 4 were obtained by adding GDL to the WPI solution (0.2 g GDL per g of protein) and stirring for 15 minutes. For rheological characterization, the solution was then poured into rheometer cup for subsequent measurements.

6.2.3 RHEOLOGICAL MEASUREMENTS

Rheological measurements were conducted using an ARES rheometer (TA Instruments, New Castle, DE) fitted with a couette geometry (cup diameter = 27 mm, bob diameter = 25mm, bob length = 32mm). Samples were covered at all times with n-hexadecane oil to prevent dehydration. All rheological tests were done at 25°C.
6.2.4 SIZE EXCLUSION CHROMATOGRAPHY (SEC)

Waters 2690 Separation Module (Waters, Milford, MA) attached to a photo diode array detector (PDA) (Waters 966) was used for chromatographic characterization. A series of 3 columns (Waters Ultrahydrogel 250, 500 and 2000) was used to separate molecular weights in the range of (10 kDa to 7,000 kDa). The eluting solvent consisted of 10 mM Tris buffer, 100 mM NaCl and 0.02% sodium azide. Flow rate through the SEC columns was maintained at 0.5 ml/min in all the runs.

6.3 RESULTS AND DISCUSSIONS

Protein solutions of concentrations of 5% and 7% were heated at 80 °C for 1 hr to denature the protein and induce disulphide bonds. Transglutaminase was then added to induce ε-(γ-glutamyl)lysine bonds to the denatured protein by incubation at 50 °C for 12 hrs. Four types of interactions/bonding are expected to take place during the thermal and enzymatic treatment: hydrophobic interactions, hydrogen bonds, disulfide bonds and ε-(γ-glutamyl)lysine bonds. We examine the relative importance of each of these interactions in subsequent sections starting with the properties of native protein.

6.3.1 CHARACTERIZATION OF NATIVE PROTEIN

Characterization of molecular size of native whey proteins was done through size exclusion chromatography. Figure 1 shows the chromatograms of native whey protein isolate sample, in which the two main peaks correspond to β-lactoglobulin (β-LG), and α-lactalbumin (α-LA). The dashed chromatogram represents native sample that underwent complete denaturation, i.e., after treatment with urea, SDS and DTT. These reagents were used to dissociate hydrogen bonding, hydrophobic interactions and disulphide bonding, respectively (17). The dashed chromatogram shows that the
dissociation of these interactions causes β-LG and α-LA to be indistinguishable. The α-LA peak moves to earlier elution time because the molecular size increases due to its change in conformation from a globular compact one into an extended one. For β-LG, two competing possibilities exist: (1) the dissociation of the dimeric state in the native state, which is known to occur at neutral conditions \((18,19)\), into a monomeric state, that can lead to smaller size and longer elution time, and, (2) the transformation of globular conformation into an extended polymer. It is evident from the Figure 1 that the first factor is dominating the behavior as the peak of β-LG elutes at a longer time than the native peak.

6.3.2 THERMALLY INDUCED WHEY PROTEIN POLYMERS

*Role of hydrogen bonding.* Figures 2a and 3a are chromatograms of preheated whey protein solutions showing the effect of different reagents on the size of polymerized proteins (also referred to as aggregates in this paper) at two different concentrations (7% and 5%, respectively). Urea (8 M) was added to study the effect of the hydrogen bonding. We observe the major peak to show slight (i) shift to longer times and/or (ii) weakening in intensity in the presence of urea, suggesting minor dissociation of the polymers. Hydrogen bonding therefore plays a small role in building the protein polymer during thermal treatment. This result agrees with the observation of Hoffman et al (1997) \((18)\) who found little effect of hydrogen bonding on the molecular weight of β-LG upon addition of 6 M urea. We have to remember though that the polymers passing through the size exclusion columns are very dilute, and hence we may expect hydrogen bonding to be stronger in the actual concentrated solutions. Other studies showed an appreciable
presence of hydrogen bonding between the aggregates using Raman spectroscopy or infrared spectroscopy (20-23).

**Role of hydrophobic interactions.** To examine the role of the hydrophobic associations, we further added 2% SDS to the protein solution that had already been treated with 8 M urea. The addition of SDS does not shift the main aggregates peak, but decreases the intensity of the peak slightly indicating dissociation of a few large aggregates into smaller ones. This indicates that hydrophobic associations also do not play a major role in the polymerization or aggregation of the whey protein upon heating, similar to the effect of hydrogen bonding.

**Role of disulfide bonding.** The additions of disulfide cleaving agent (DTT) in the presence of urea and SDS causes dramatic change to the chromatogram, dissociating all the large aggregates into monomers. The presence of a single peak rather than two peaks is similar to what we found and discussed in Figure 1. This dramatic dissociation indicates that disulfide linkage is a major factor responsible for formation of the protein polymer upon thermal treatment. The chromatograms indicate complete dissociation of aggregates to the monomer level, although it has been reported elsewhere that in some cases (18), addition of DTT did not break all the disulfide bonds to the monomer level. The differences may be due to the concentration of DTT, presence of the other dissociating agents, different pH and protein concentration.

6.3.3 CROSSLINKING BY TRANSGlutaminase AND THE ROLE OF ε-(γ-Glutamyl)Lysine Bonding

**Figures 2b and 3b** show the chromatograms of whey protein samples, preheated at 80 °C for 1 hr and then incubated with transglutaminase (10 U/gm protein) for 12 hrs at
80 °C. The addition of urea and SDS shows almost no decrease in the molecular weight, unlike the slight effect of urea or SDS on the thermally-induced whey protein polymers without enzymatic treatment. This behavior indicates that the extent of the hydrogen bonding and hydrophobic interactions are different in the cases. This may be due to the shape of the protein polymer molecule with and without enzyme treatment. Disulfide bonded whey proteins are known to be linear in shape, while we expect the enzymatically crosslinked polymers to be branched. Branching may cause steric hindrance that decreases the extent of hydrogen bonding or hydrophobic interactions. In our previous work (24) using infrared spectroscopy, we found differences in the CH stretching mode for β-lactoglobulin upon treating with enzyme, suggesting changes in the hydrophobic associations. Finally, upon addition of all the denaturing and disulfide cleaving agents, the molecular weight gets even smaller and we find a wide distribution of polymers (aggregates) with high concentration species eluting at 54 minutes. This peak suggests molecular weight equivalent to a mixture of monomers and dimers of β-LG and α-LA as observed in Figure 1. Perhaps the most intriguing part of the chromatogram is the occurrence of a wide shoulder reflecting the presence of a broad spectrum of species including high molecular weight ones. These polymers or aggregates resulting from enzymatic crosslinking are bonded together only the by ε-(γ-glutamyl)lysine bonds as the other associations have been annulled using urea, SDS and DDT.

**Effect of enzyme concentration.** Figure 4 shows chromatograms of whey proteins samples crosslinked at two different concentrations of transglutaminase, 10 and 50 U/gm protein. We see almost no effect on the molecular weights of the aggregates as we increase enzyme concentration from 10 to 50 U/gm (chromatograms to the right).
However, we clearly see on the same graph that in the absence of disulfide bonds, hydrogen bonds and hydrophobic associations, the aggregates obtained with 50 U/g enzyme are significantly larger than that obtained with 10 U/g enzyme. This observation indicates that the new bonds formed due to the higher enzyme concentration are primarily intramolecular crosslinks. We can also observe that the monomeric/dimeric form of β-LG and α-LA is still the most abundant species; however, multimodality starts to appear, decreasing the monomer/dimer peak height. Similar trend was observed at 3% and 7% protein concentration (data not shown).

**Effect of protein concentration.** Figure 5 shows the effect of protein concentration on the molecular weight distribution of protein samples preheated and then crosslinked with transglutaminase (10 U/gm). The molecular weight increases considerably with concentration. This cannot be explained based merely on the enzymatic crosslinking reaction because the preheating step, which induces disulfide polymerization, is concentration dependent as well. So, we treated the aggregates with urea, SDS and DTT to dissociate all bonds except ε-(γ-glutamyl)lysine bonds. The molecular weight distribution looks similar for the two concentrations, with the major peak eluting at 54 minute, indicating that the predominance of the monomeric/dimeric species. The similarity in the molecular weights at the two concentrations indicates that enzymatic reaction at these concentrations may be in zeroth order so that the rate of the reaction is independent of protein concentration.

**6.3.4 COLD-SET GELATION**

The previous section delineated the role of each type of interactions in building the protein polymers/aggregates in solution. In this section, we study the role of each type
of these interactions in the formation of acidic cold set-gels at pH 4. We induce gelation by slow acidification using GDL, at ambient temperature. The amount of GDL needed was found to be linearly dependant on protein concentration (0.2 gm GDL per gm of protein). The gel reaches the required pH (4 ± 0.1) in ~ 24 hrs. In order to examine the effect and role of each type of interactions, we used urea, SDS and β-ME to break hydrogen bonds, hydrophobic associations and disulfide bonds, respectively. β-ME was used instead of DTT for cost issues and to facilitate mixing with the protein solution when inducing gelation by GDL.

Physical interactions are known to be a primary factor in cold set gelation of whey proteins (8,25), however, the exact role of hydrophobic interactions and hydrogen bonding on gel rheology are not yet understood.

*Role of hydrophobic interactions.* Figure 6 shows the evolution of elastic modulus (G’) with time for samples without enzyme treatment with different amounts of SDS added. The viscous modulus is of much lower value and is not shown for the sake of clarity. We observe a large difference in the gelation behavior upon addition of SDS. The elastic modulus reaches a maximum and then drops drastically by one or two orders of magnitude, the extent of reduction being directly related to the amount of SDS. When the samples were visually examined, we found fracture surfaces and syneresis, explaining the unexpected decrease in G’. This indicates that hydrophobic interactions are crucial for building the gel network and holding the water in the gel matrix. The syneresis in the gel can be explained by the fact that hydrophobic associations enhance the solubility of the non-polar species according to Shinoda (26). Once these hydrophobic associations are screened, the solubility of protein molecules decrease and syneresis occurs. Figure 7
shows the elastic modulus as a function of strain for samples with and without SDS. The samples with SDS show no linear viscoelastic region in the examined strain range, indicating immediate network disruption. The effect is more pronounced at the higher SDS concentration. This behavior emphasizes the major role of hydrophobic interactions in sustaining the stress or strain applied to the gel. It is worth mentioning that addition of SDS decreases the acidification effect of GDL. Without SDS, 0.2 g GDL/g protein was needed to change 7% WPI solution from pH 7 to pH 4 in 24 hrs, whereas with 2% SDS, 0.44 g GDL/g needed was needed. Higher concentrations of SDS (4% and 10%) were also used with the protein (data not shown), and surprisingly, stronger gels were obtained with no syneresis or cracks. However, gel properties at these conditions (i.e., at higher SDS concentrations) are believed to be affected by the formation of SDS micelles; allowing protein hydrophobic residues to form additional number of active intermolecular junctions. Such behavior has been observed in hydrophobically modified associative polymers (27). Consequently, the interpretation of data at high SDS concentrations cannot be simply related to the decrease in hydrophobic interactions.

The frequency spectra of the elastic (G’) modulus of preheated samples with and without SDS are shown in Figure 8. A representative G’’ plot is shown for one sample for the sake of clarity. In all cases though G’’ is much lower than G’ with G’ relatively independent of frequency, suggesting gel behavior. However, the magnitude of G’ decreases considerably upon addition of SDS. It is interesting to note though that even without the presence of hydrophobic associations a three dimensional gel network, albeit fragile, is formed.
**Role of hydrogen bonding.** We used 8 M urea to disrupt hydrogen bonding immediately before addition of GDL. The disruption of hydrogen bonding prevented the gelation at these conditions and the whey protein obtained at pH 4 was in solution form. This behavior indicates that breaking the hydrogen bonds is detrimental in cold set whey protein gels. Although the enthalpy of formation of a hydrogen bond is only around 5-10 \( k_B T \) (28) (where \( k_B \) is Boltzman constant and \( T \) is the absolute temperature) per hydrogen bond at room temperature, they are vital in building the gel network due to the large number of bonds along the backbone. It is worth mentioning that the presence of urea required more GDL to reach a pH of 4. The amount of GDL needed to acidify 7% whey protein from pH 7 to pH 4 increased from 1.4% with no urea to 2.7% with 8 M urea.

**6.3.5 CHEMICAL BONDING**

Disulfide bonds were cleaved by \( \beta \)-ME addition (4%) immediately before acidulation by GDL. **Figure 9** shows the evolution of \( G' \) with and without \( \beta \)-ME for heat and heat+enzyme treated samples. Considering only the heat treated sample, we find that that cleavage of the disulfide bonds stabilizes \( G' \) against the decrease with pH. The decrease in \( G' \) with time for the preheated only sample has been observed in our earlier work (11) and attributed to the decrease of pH beyond the isoelectric point. The presence of additional enzyme catalyzed \( \varepsilon-(\gamma\text{-glutamyl}) \)lysine bonds to the heat treated samples stabilizes the sample to some extent. In this case too, presence of \( \beta \)-ME further stabilizes \( G' \) to pH changes. In fact, cleavage of the disulfide bonds leads to the same result for both heat or heat+enzyme treated samples. The stability upon addition of \( \beta \)-ME may be interpreted on the basis of the increased extent of physical interactions upon cleaving the
disulfide bonds. The increased exposure of hydrophobic residues may lead to more physical interactions and hence more stability in the gel structure.

It is interesting to note that the G’ values in Figure 9 are close to each other for the different samples, unlike the observed decade differences when screening the hydrophobic interactions (Figure 6) or the inhibition of gelation when screening the hydrogen bonding. This indicates that gel rigidity, manifested in the value of G’, is mildly affected by the size of the starting polymer (i.e., extent of chemical bonding). This is not unexpected as the starting polymer prior to acidulation is a solution and not a gel and it is the subsequent physical junctions that lead to gel formation.

The frequency spectra of the elastic modulus of the samples following acidulation is shown in Figure 10. Consistent with Figure 9, we find G’ of both samples (heat treated only and heat + enzyme treated) to be the same in the presence of β-ME. In addition, all samples have the same frequency dependence suggesting similar relaxation behavior. However, the G’ of the preheated sample is lower in the absence of β-ME than in its presence, indicating that the presence of disulfide linkages has a deleterious effect on gel modulus. In contrast, disulfide bonds are of crucial importance for large strain behavior. This is illustrated in Figure 11 which plots G’ as a function of increasing strain. The fracture strain, corresponding to the point of decrease in G’, is substantially lower in the absence of disulfide bonds than in its presence. Such behavior indicates that the disulfide bonds play a major role in the fracture properties of the gels. Figure 11 also shows that presence of enzymatic crosslinks recovers to a large extent the brittleness caused by disulfide cleavage using β-ME.
6.4 CONCLUSIONS

In this study, we examined the role of various chemical and physical bonds in the formation of whey protein gels using a two step process involving initial polymerization and subsequent cold-set acidic gelation. During the first step, chemical bonds play a dominant role in building the whey protein polymer with some participation of the hydrophobic interactions and hydrogen bonding. Enzymatic crosslinking of whey proteins changed the extent of the hydrophobic interaction as explored by the effect of SDS on the samples with and without enzyme treatment. The highest concentration of protein molecules after crosslinking with transglutaminase - after dissociation with urea, SDS and DTT - are for monomer/dimer species. The properties and formation of cold-set gels, are primarily dependent on hydrophobic interactions and hydrogen bonding. The absence of hydrophobic interactions led to a fragile network and syneresis while gelation was completely prohibited by hydrogen bonding dissociation using urea. The complete absence of chemical bonds did not prevent gelation and had a mild effect on the elastic modulus of the gel. However, chemical bonds have a profound effect on gel strength and flexibility. Gels with no disulfide bonding fractured at much smaller strain values compared with gels with disulfide bonds. The introduction of ε-(γ-glutamyl)lysine bonds for samples with no disulfide bonds restored the strength and flexibility of the gels.
6.5 REFERENCES


Figure 1. Chromatogram of whey protein isolate sample. Continuous chromatogram represents native sample with no additives, while dashed chromatogram represents sample with urea, SDS and DTT.
Figure 2. Chromatograms of 7% whey protein isolate samples. (a) Thermally treated at 80 °C for 1 hr, (b) Thermally treated as (a) but further incubated with transglutaminase enzyme (10U/g) at 50 °C for 12 hrs.
Figure 3. Chromatograms of 5% whey protein isolate samples. (a) Thermally treated at 80 °C for 1 hr, (b) Thermally treated as (a) but further incubated with transglutaminase enzyme (10U/g) at 50 °C for 12 hrs.
Figure 4. Chromatograms of 5% whey protein isolate samples, thermally treated at 80 °C for 1 hr, then incubated with transglutaminase enzyme at two different concentrations 10U/g and 50U/g.
Figure 5. Chromatograms of 5% and 7% whey protein isolate samples, thermally treated at 80 °C for 1 hr, then incubated with transglutaminase enzyme.
Figure 6. Evolution of elastic modulus ($G'$) of preheated 7% whey protein samples after addition of 1.4% GDL, showing the effect of SDS addition. Experiments were done at 25 °C, frequency of 1 rad/sec and strain of 0.5%.
Figure 7. Elastic modulus ($G'$) as a function of strain of preheated 7% whey protein samples showing the effect of SDS addition. Experiments were done at 25 °C and frequency of 1 rad/sec
Figure 8. Mechanical spectra of preheated 7% whey protein samples after addition of 1.4% GDL, showing the effect of SDS addition. Experiments were done at 25 °C and strain of 0.5%.
Figure 9. Evolution of elastic ($G'$) modulus of preheated 7% whey protein samples after addition of 1.4% GDL showing the effect of chemical bonding. Experiments were done at 25 °C, frequency of 1 rad/sec and strain of 0.5%.
Figure 10. Mechanical spectra of preheated 7% whey protein samples after addition of 1.4% GDL, showing the effect of chemical bonds. Experiments were done at 25 °C and strain of 0.5%.
Figure 11. Elastic modulus (G') as a function of strain of preheated 7% whey protein samples showing the effect of chemical bonds. Experiments were done at 25 °C and frequency of 1 rad/sec.
CHAPTER 7
ENZYMATIC CROSSLINKING OF β-LACTOglobulin: CONFORMATIONAL PROPERTIES USING FTIR SPECTROSCOPY

A revised version of chapter 7 by Ahmed S. Eissa, Christa Puhl and Saad A. Khan will be submitted to the *Journal of Agricultural and Food Chemistry.*
Enzymatic Crosslinking of β-lactoglobulin: Conformational Properties Using FTIR Spectroscopy

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ABSTRACT

FTIR spectroscopy is used to probe the conformational properties of β-lactoglobulin subjected to enzymatic crosslinking using transglutaminase. Spectra in the amide I region (1600-100 cm⁻¹) of denatured conformations of β-lactoglobulin, known to be necessary for crosslinking, differ according to the denaturation procedure. Denaturation by chemical denaturants, dithiotheritol or β-mercaptoethanol, shows no effect on the α-helix, while shifting the monomer to dimer equilibrium towards a higher monomer concentration. On the other hand, denaturation by thermal treatment dissociates the β-sheets in the native structure, forming new intermolecular β-sheets. Enzyme crosslinking of chemically-denatured β-LG changes the structure of the α-helix and induces intermolecular β-sheets. In contrast, enzyme crosslinking of thermally-denatured β-LG molecules produce no change in the spectra of the amide I region but exhibit a different spectra in the CH stretching mode region (2800-3100 cm⁻¹), indicating a change in the hydrophobic interactions between protein molecules.

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7.1 INTRODUCTION

Whey proteins are an important food ingredient of high nutritional value and unique functional properties (1-3). The functional attributes include gelation, emulsification, foaming and thickening and depend primarily on the interactions between the protein molecules and/or between the proteins and solvent. These interactions, in turn, are influenced by the chemical, structural and conformational properties of the protein molecules, particularly β-lactoglobulin as it forms the major constituent of whey proteins.

Enzymatic crosslinking of whey proteins provides a powerful tool to tailor their functional properties. In this regard, transglutaminase enzyme has been widely used to crosslink proteins (4-18). In these research studies, workers investigated the polymerization, rheology, texture and microstructure of protein solutions and gels after the crosslinking with transglutaminase. Transglutaminase proved to increase protein molecular weight considerably (14,15), enhance gel modulus, increase gel fracture strain and stress (15) and improve film properties (19). However, little effort has been made to examine the conformational characteristics of protein molecules and their effect on transglutaminase catalyzed reaction.

An investigation of the conformational characteristics of β-lactoglobulin (β-LG), the main constituent of whey proteins, is essential to help understand its susceptibility for being crosslinked by enzymes. For instance, the secondary and tertiary structures of β-LG in the native state may impede enzymatic catalysis. Consequently, complete or partial denaturation of β-LG may be necessary for enzymatic reactions. In fact, prior work in our laboratory (15) using transglutaminase to crosslink whey proteins revealed that
denaturation is necessary to crosslink β-LG but not α-lactalbumin, which can readily crosslink in its native state. However, it is not yet fully understood what are the critical conformational changes that occur to the β-LG molecule allowing for enzyme crosslinking. Of equal importance are the conformational changes that result from the enzymatic crosslinking.

In this study, we address the conformational changes required to facilitate the crosslinking of β-LG by transglutaminase and the resulting conformational changes accompanied with the crosslinking. A required step prior to enzyme crosslinking is the denaturation of β-LG. Using FTIR as a tool, we therefore examine how/if different modes of denaturation, heat versus chemical treatment, affect protein conformation and subsequent enzyme crosslinking.

7.2 MATERIALS AND METHODS

7.2.1 MATERIALS

β-lactoglobulin (>99%), N-ethylmaleimide (NEM), β-mercaptoethanol (β-ME), and dithiotheritol (DTT) were obtained from Sigma Chemical Co. (St.Louis, MO). Deuterium oxide (D₂O) was obtained from Aldrich (Milwaukee, PA). A commercial version of transglutaminase enzyme (1% Enzyme and 99% Maltodextrin, by weight) was supplied by Ajinomoto Co., Japan.

7.2.2 PREPARATION OF PROTEIN SOLUTION

β-lactoglobulin was dissolved in deuterium oxide at concentrations of 3%, 5%, and 7% by weight. Samples were purged with nitrogen and kept under nitrogen atmosphere for 48 hrs to complete H/D exchange. Some samples were heated at 80 °C for 1 hr to denature the protein. Other samples were denatured by treatment with DTT (50
mM). For samples treated with enzyme, the enzyme was added (50 U/gm) and then thoroughly shaked. The sample was then incubated at 50 °C for 12 hrs. The enzyme was found to lose activity completely after ~12 hrs.

7.2.3 FTIR MEASUREMENTS

Infrared spectra were recorded using Magna 760 Nicolet spectrometer (Madison, WI). Dry air was continuously run through the spectrometer. The infrared spectra were recorded at a resolution of 2 cm⁻¹. A total of 128 scans were recorded, averaged and apodized with Happ-Genzel function. Deconvolution of the spectra (1600 cm⁻¹ to 1700 cm⁻¹) was performed by Ominc 5.2 software.

7.3 RESULTS AND DISCUSSIONS

7.3.1 SPECTRA OF NATIVE β-LG MOLECULE

The FTIR spectra of β-LG has been a subject of numerous studies (20-30) with special attention to the spectral range of 1600 to 1700 cm⁻¹. This region is known as the Amide I region (31) and corresponds mainly to the peptide backbone C=O stretching mode in addition to the C-N stretching mode. Bands in this region correspond to β-sheets, α-helix, turns, and random coils. Figure 1 shows a three dimensional structure of β-LG. Under physiological conditions, β-LG exists as a dimer (32). Secondary structure of β-LG under these conditions consist of nine β-strands (~50%), single α-helix (10~15%), turns (~20%), and random arrangements (~15%) (33). The spectra of the native β-LG molecule at different concentrations showing the different components (bands) are revealed in Figure 2. The bands at 1621 cm⁻¹, 1634 cm⁻¹ and 1692 cm⁻¹ correspond to β-sheets, while that at 1649 cm⁻¹ represents α-helix. The band at 1677 cm⁻¹ corresponds to turns or β-sheets, and the one at 1663 cm⁻¹ corresponds to turns. The small shoulder
appearing at 1605 cm\(^{-1}\) reflects side chain residues. It is clear from Figure 2 that the protein concentration in the range shown (3\% to 7\% by weight) has very little influence on the position of the bands. However, it has been reported elsewhere that at lower concentrations (less than 1\%) some bands change dramatically (25). Lefevre and Subirade (25) showed complete disappearance of the bands at 1621 cm\(^{-1}\) and 1692 cm\(^{-1}\), while the band at 1635 cm\(^{-1}\) was shifted to 1629 cm\(^{-1}\) at a concentration of 0.25\%. This indicates that the bands do not correspond merely to the secondary structures of the individual molecules, but also depend to a certain extent on the interaction between the molecules. The change of the spectrum with concentration was attributed to the monomer to dimmer ratio in the protein solution (25). This ratio changes with protein concentration and hence the bands change from one concentration to another. However, this behavior seems to be dependent on the range of concentration of interest as we see no difference in the spectra in the range of 3\% to 7\%.

7.3.2 CROSSLINKING OF \(\beta\)-LG BY TRANSGLUTAMINASE ENZYME

Transglutaminase enzyme crosslinks \(\beta\)-LG molecule by inducing the acyl transfer reaction between the lysine and glutamine residues, producing \(\varepsilon\)-\((\gamma\text{-glutamyl})\)lysine bonds (34). Such bonds cause aggregation of the protein and lead to high molecular weight polymers. However, previous work by us (15) indicates that \(\beta\)-LG molecule must be partially or completely denatured to undergo enzymatic crosslinking. The denaturation can be induced by thermal treatment at a temperature higher than the denaturation temperature or by addition of denaturants such as dithiotheritol (DTT) or \(\beta\)-mercaptaethamol (\(\beta\)-ME) that cleave the disulfide bonds. Figure 3 shows an electrophoresis gel for different \(\beta\)-LG samples that were incubated with transglutaminase
enzyme for 12 hrs at 50 °C. The temperature of incubation was chosen as it has been shown to be the optimum temperature for enzyme activity (35), while the duration (12 hrs) was chosen based on enzyme activity (the enzyme become inactive after 12 hrs at 50 °C). The heat denatured sample (80 °C for 1 hr, lane 4) and the samples treated with chemical denaturants (DTT and β-ME, lanes 5 and 6, respectively) were polymerized (i.e., crosslinked) by the enzyme to form large molecular weight aggregates and appear on the top of the gel. However, the native β-LG sample (lane 3) remains unpolymerized. This behavior indicates that crosslinking by transglutaminase requires denaturation of β-LG either thermally or by chemical denaturants such as DTT or β-ME. We believe that this behavior is primarily due to the globular nature of β-LG, which has deep hydrophobic pockets. However, the mechanism of thermal denaturation is quite different from the mechanism of denaturation induced by chemical denaturants that cleave disulfide bonds, as discussed in the subsequent section.

7.3.3 STRUCTURAL CHANGES UPON β-LG DENATURATION

7.3.3.1 THERMAL DENATURATION

During thermal treatment, the hydrogen bonds stabilizing the native structure of β-LG are disrupted, causing loss of the α-helix and β-sheets structures and creating new β-sheets arrangements. These new β-sheets result from the intermolecular hydrogen bonding between the protein aggregates. Aggregation is induced primarily by disulfide interchange and hydrophobic interactions (36,37). Figure 4 shows Fourier self deconvoluted spectra of β-LG samples denatured by thermal treatment (i.e., heating to 80 °C for 1 hr). Comparison of Figure 4 with Figure 1 shows that thermal treatment diminishes the bands at 1621 cm⁻¹, 1634 cm⁻¹ and creates a strong band at 1612 cm⁻¹,
indicative of intermolecular, strong β-sheets structures (38). We can also observe that heat treatment diminishes the bands at 1649 cm\(^{-1}\) and 1692 cm\(^{-1}\) corresponding to α-helix and β-sheets, respectively, and creates a band at 1682 cm\(^{-1}\) indicative of antiparallel β-sheets formation (31).

### 7.3.3.2 CHEMICAL DENATURATION

Denaturation by DTT and β-ME, on the other hand, proceeds quite differently as they disrupt β-LG structure by cleaving the two disulfide bonds present in the native molecule: (Cys66-Cys160) that connects C-D loop to the carboxyl-terminal region, and (Cys106-Cys119) that links strands G and H as shown in Figure 1. When using DTT at specific β-LG concentration, the spectrum depends to large extent on the concentration of DTT (data not shown). We used excess of DTT (500 mM) to ensure complete denaturation of β-LG. Upon using DTT, the bands at 1621 cm\(^{-1}\) and 1634 cm\(^{-1}\) (Figure 5) move closer to each other and an intermediate band appears at 1629 cm\(^{-1}\). In addition, the band at 1692 cm\(^{-1}\) completely disappears. Similarly, the addition of β-ME produces the same changes, with an intermediate band appearing at 1626 cm\(^{-1}\) and the band at 1692 cm\(^{-1}\) vanishing entirely. This phenomenon can be attributed to the rearrangement of the β-sheets after the cleavage of the disulfide bonds. The disappearance of the 1634 cm\(^{-1}\) band may be interpreted as a decrease in the dimer content of β-lactoglobulin (39). This is confirmed by the fact that (Cys106-Cys119) is involved in dimer formation of β-lactoglobulin (40). In both DTT and β-ME, the band at 1649 cm\(^{-1}\) effectively remains unchanged, indicative of minimal change to the α-helix structure. The bands at 1663 cm\(^{-1}\) and 1677 cm\(^{-1}\) also remain almost unchanged. Hereby, the spectra of denatured β-LG by DTT and β-ME indicate minimal change of α-helix while the major changes occur in the
β-sheets structures, mainly in the bands at 1621 cm\(^{-1}\), 1634 cm\(^{-1}\) and 1692 cm\(^{-1}\). This agrees with the fact that such denaturants cleaves disulfide bonds, which do not interact with the α-helix. The cleavage of the disulfide (Cys66-Cys160) will disconnect the C-D loop from the carboxyl-terminal region (see Figure 2), which is expected to open up the protein globule, as this bond attaches 2 distant amino acids. We also expect the C-D loop to participate in other β-sheet associations, possibly explaining the rearrangements of the bands at 1621 cm\(^{-1}\) and 1634 cm\(^{-1}\). On the other hand, the cleavage of (Cys106-Cys119) will weaken the contact between strands G and H. The extent of this weakening and weather it is going to disrupt the antiparallel arrangement of strands G and H is not known and has not been addressed before. Intuitively, we can postulate that (Cys66-Cys160) will have more effect in opening the structure compared with (Cys106-Cys119) according to the geometry of the molecule. However, an exact knowledge of the conformational changes corresponding to cleavage of each disulfide bond requires careful molecular modeling and is beyond the scope of this work.

### 7.3.3.3 THERMAL DENATURATION IN THE PRESENT OF CYSTEINE BLOCKING AGENT

Chemical reagents blocking the cysteine residues in β-LG molecules prevent disulfide interchange reactions. We used N-ethylmaleimide (NEM), a typical cysteine blocking reagent, to study the thermal denaturation in the absence of disulfide interchange. Figure 6 shows FTIR spectra of β-LG samples, preheated in the absence or presence of NEM. In the presence of NEM, we notice the aggregate band shifts to 1620 cm\(^{-1}\) compared with 1612 cm\(^{-1}\) in the absence of NEM. The shift to a higher wavenumber may be explained in terms of the presence of weaker β-sheets, compared with the
unblocked sample. Compared with the native spectrum, we find the band at 1634 cm\(^{-1}\) disappears in the preheated sample, regardless whether NEM is present or not, indicating that the dissociation of the \(\beta\)-LG dimer does not depend on the disulfide interchange reaction. In contrast, the band at 1682 cm\(^{-1}\) that appears in the absence of NEM exhibits a slight shift to 1680 cm\(^{-1}\). The large band at 1694 cm\(^{-1}\) that appears in the sample with NEM is due to the imide group of the NEM because a separate run with NEM in solution shows a single strong peak at 1692 cm\(^{-1}\) (data not shown). From our preceding discussion, we conclude that the main factor facilitating the crosslinking of \(\beta\)-LG by transglutaminase is the disruption of the \(\beta\)-sheets structures. The shift or disappearance of the band at 1634 cm\(^{-1}\) under denaturing conditions indicates that the presence \(\beta\)-LG as a dimer may be another factor impeding the crosslinking.

7.3.4 SPECTRA OF CROSSLINKED \(\beta\)-LG MOLECULES

The treatment of \(\beta\)-LG by transglutaminase enzyme is expected to cause some structural changes and affect the FTIR spectra. **Figure 7** shows FTIR spectra of \(\beta\)-LG after thermal denaturation and after enzymatic treatment following preheat. We observe no difference in the two spectra between heat and heat+enzyme treatment in the amide I range. This indicates that C=O stretching is not affected by the enzymatic crosslinking although the crosslinking reaction changes the microenvironment around the carbonyl side chain of the glutamine that reacted with lysine. This similarity in spectra may be attributed to the low number of the bonds created with respect to the backbone bonds in the protein. However, in the case of chemical denaturation by DTT (**Figure 8**), we clearly observe a noticeable diminution in the \(\alpha\)-helix band (at 1650 cm\(^{-1}\)) upon crosslinking by transglutaminase. The \(\alpha\)-helix of \(\beta\)-LG molecule contains two lysine
residues. So, the alternation of the spectrum is possibly due to the crosslinking of one or both of the lysine residues. In addition, an intense band appears at 1613 cm⁻¹, indicative of intermolecular β-sheets resulting from aggregation. It is worth mentioning here that the sample with DTT with no enzyme was subjected to a similar thermal treatment (50 °C for 12 hrs) to isolate the effect of heating from that of enzyme crosslinking. To summarize, crosslinking of β-LG does not change the amide I spectra of the preheated protein while in the case of chemical denaturation, crosslinking changes the α-helix pattern and creates intermolecular β-sheets due to protein aggregation.

7.3.4.1 INVESTIGATION OF THE C-H STRETCHING MODE AFTER CROSSLINKING WITH ENZYME

Hydrophobic interactions play an important role in β-LG aggregation. We therefore examined the C-H stretching mode, typically in the spectral range of 2800 - 3100 cm⁻¹ corresponding to diversity of CH, CH₂, and CH₃ in the side chains of the amino acids. The main band at 2940 cm⁻¹ for the protein was reported to represent aromatic, aliphatic and other amino acids (41). Bands around 2900 cm⁻¹ and 2880 cm⁻¹ primarily represent aliphatic amino acids. Figure 9 compares the spectra of native, preheated and preheated then enzyme treated β-LG samples in the above-mentioned range. We can clearly observe a change in the bands at 2938 cm⁻¹ and 2962 cm⁻¹ between the samples with and without enzyme treatment. Assignments of each of these two bands may be interpreted based on the spectra of the individual amino acids reported elsewhere (41), as follows. The band around 2960 cm⁻¹ corresponds to alanine, cystine, histidine, serine, leucine and tryptophan, while the band around 2940 corresponds to 16 different amino acid residues. The change in the spectra upon enzymatic treatment indicates that
the reaction between lysine and glutamine changes the hydrophobic interactions to some extent. The lysine and glutamine residues contain 4 and 2 methylene groups in their side chains, respectively. Enzymatic treatment may have changed the microenvironment around the hydrophobic groups in the molecules, thus giving a different hydrophobic interaction pattern.

7.4 CONCLUSIONS

In this study, we used FTIR analysis to examine the conformation characteristics of β-lactoglobulin subjected to enzymatic crosslinking. We explored the conformation during both the denaturation step and the subsequent enzyme-catalyzed polymerization stage. β-LG molecule needs to be denatured either by heat or chemicals (i.e., cleaving of disulfide bonds) to be susceptible to crosslinking by transglutaminase enzyme. We found thermal denaturation to proceed quite differently from chemical denaturation, as manifested in the spectra of the amide I region. Denaturation by chemical denaturants, dithiotheritol or β-mercaptoethanol, showed no effect on the α-helix but shifted the monomer to dimer equilibrium towards a higher monomer concentration, whereas denaturation by thermal treatment dissociated the β-sheets in the native structure and led to formation of new intermolecular β-sheets.

Crosslinking by transglutaminase of the thermally denatured β-LG did not change the spectra in the amide I region indicating that the carbonyl stretching mode is unaffected by subsequent enzyme crosslinking. In contrast, enzyme crosslinking of chemically denatured β-LG changed the structure of the α-helix and induced intermolecular β-sheets. By investigating the C-H stretching mode however, we found that crosslinking by transglutaminase of thermally denatured β-LG produced some
changes in the hydrophobic interactions that affected the spectra in the C-H stretching region.
ACKNOWLEDGMENTS

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7.5 REFERENCES


Figure 1. Schematic representation of a three-dimensional structure of β-lactoglobulin molecule showing β-strands, α-helix, the position of the disulphide bonds, and free cysteine residue. (reproduced with permission from(42))
Figure 2. FTIR spectra of native β-lactoglobulin at concentrations of 3, 5 and 7%.
Figure 3. SDS-PAGE of β-LG samples under reducing conditions. Lane 1: molecular weight markers, lane 2: native β-LG, lane 3: enzyme-treated native β-LG sample, lane 4: enzyme-treated β -LG sample that was preheated for 1 hr at 80 °C, lane 5: enzyme treated β-LG sample in the presence of DTT, and, lane 6: enzyme treated β-LG sample in the presence of β-ME. Enzyme treatment was done by incubation of the sample at 50 °C with transglutaminase enzyme (10 U/g protein) for 12 hrs.
Figure 4. FTIR spectra of preheated (80 °C for 1 hr) β-lactoglobulin at concentrations of 3, 5 and 7%.
Figure 5. FTIR spectra of native, DTT treated, and BME treated β-lactoglobulin samples, all at a concentration of 7%.
Figure 6. FTIR spectra of native, preheated and preheated with NEM β-lactoglobulin samples at 7% concentration.
Figure 7. FTIR spectra of preheated (solid curves), and enzyme treated (dashed curves) β-lactoglobulin samples at concentrations of 3, 5 and 7%.
Figure 8. FTIR spectra of native, DTT treated, and DTT followed by enzyme treated β-lactoglobulin samples at 7% concentration. The sample with DTT was heated for 12 hrs at 50 °C to mimic the enzymatic incubation procedure.
Figure 9. FTIR spectra of native, preheated and preheat followed by enzyme treated β-lactoglobulin samples at 7% concentration.
CHAPTER 8

A PROBABILISTIC MODEL OF THE POLYMERIZATION OF

β–LACTOglobulin BY TRANsGLUTAMINASE ENZYME
ABSTRACT

A polymerization model has been developed for β-lactoglobulin polymerization by transglutaminase enzyme prior to gel point. The model was based on the stepwise polyfunctional polymerization using the probabilistic approach. According to the model, gelation starts at glutamine conversion of 5.8%. Experimental procedure for model verification is suggested as a future extension of this work.
8.1 INTRODUCTION

Whey proteins are important food ingredients that are used in a number of food products such as desserts, dairy products and confectionary items and desserts (1,2). The utilization of whey proteins has been an important research focus over the past few decades because of their abundance and high nutritional value. Manufacturer like these proteins because of their economic appeal as a value-added ingredient, technical product developers like them for their solubility and varied functionalities, and consumers for their digestibility and low caloric intake (2,3). The main ingredient of whey proteins is β-lactoglobulin, which is primarily responsible for imparting the functional characteristics by undergoing polymerization and gelation under different conditions. Typically, polymerization of β-lactoglobulin takes place under heat denatured conditions through disulphide bonding between sulfhydryl groups, and has been discussed in details in the literature (4-6). On the other hand, polymerization of β-lactoglobulin can also be catalyzed using enzymes, in particular transglutaminase which has been widely used for crosslinking proteins (7-11).

Previous work in our laboratory and elsewhere has explored several aspects of enzyme crosslinking using transglutaminase. These include developing protocols to form gels, evaluating rheological behavior, examining gel microstructure (12,13), and investigating conformational changes in the protein molecules (14). However, no effort has been made to model the polymerization reaction.

In this chapter, we introduce a model to describe the polymerization of β-lactoglobulin using transglutaminase enzyme, limiting ourselves only to crosslinking prior to gel formation. Several polyfunctional non linear polymerization models have
been proposed in the literature (15-18). Although all these models are useful in their own rights, we use as our starting point the model based on step growth polymerization developed by Macosko and Miller (17). In principle, we can obtain derivation of the molecular weight and gel point from kinetic equations, however, statistical methods provide a somewhat simpler approach (16), and hence it is more widely used in modeling of polyfunctional non linear polymerization. Using our model, we examine the effect of conversion on molecular weight and determine the extent of conversion needed to reach the gel point.

8.2 PROPERTIES AND REACTION OF β-LACTOGLOBULIN (β-LG)

β-LG consists of 162 amino acids with a molecular weight of 18.3 kDa. It is a globular protein that is found in the folded state at neutral conditions and has an isoelectric point of 5.2. Unfolding of the protein is crucial for its reactivity. Unfolded, also known as denatured, protein can polymerize due to the exposure of the reactive amino acids to the solvent. For the specific reaction induced by transglutaminase enzyme, lysine and glutamine residues (amino acids) participate in the reaction.

Transglutaminase catalyzes the acyl transfer reaction in which γ-carboxamide of the peptide-bound glutamine residues act as acyl donors. The ε-amino groups of the peptide-bound lysine act as acceptors and the result is the formation of an ε-(γ-glutamyl)lysine link as shown in Figure 1. This reaction is clearly a condensation polymerization in which ammonia is a condensation product.

8.3 MODEL DEVELOPMENT

The modeling of chain attachments is dependent on whether we are examining polymerization prior to or following gelation. In this work, we discuss polymerization in
the pregel state, which can be looked upon as a reaction between $A_f$ and $B_g$ chains. In this case, $A$ represents chains containing the glutamine residues and $B$ corresponds to the chains containing lysine residues. Subscripts $f$ and $g$ refer to the number of glutamine and lysine residues, respectively. Figure 2 shows the distribution of glutamine and lysine in the primary structure of the $\beta$-LG.

As a first step towards developing the model, we make the following assumptions:

1. All functional groups have the same reactivity.
2. All groups react independently of each other.
3. There is no intramolecular crosslinking (polymerization).

In reality, one could expect departure from these assumptions. For example, there may be some intramolecular crosslinking because the monomer (i.e., unreacted $\beta$–LG chain) is long and flexible enough to facilitate reaction of lysine and glutamine within the same chain. Nevertheless, these assumptions provide a pragmatic yet simple starting point.

Let us assume a mixture of $A_f$ and $B_g$ polymers. In our case, $f = 9$ while $g = 15$. Since $A$ is the limiting reactive group and hence we can define $r$, the stoichiometric ratio of mutually reactive groups, as

$$ r = \frac{f^o}{g^o} = \frac{9}{15} = 0.6 $$

We can also define the conversion $p$ in terms of $p = \frac{(A - A_t)}{A}$, where $A$ is the initial number of moles of group of type $A$ and $A_t$ equals the moles after some reaction time $t$. If we choose a random reactive group $A$ (glutamine), we can calculate the weight
attached to \( A \) (\( W_A^{\text{out}} \)) looking outside the molecule (as seen in Figure 3). For \( A \) (glutamine) reacting with \( B \) (lysine), we can then state:

\[
W_A^{\text{out}} = \begin{cases} 
0 & \text{if } A \text{ has not reacted} \\
 W_B^{\text{in}} & \text{if } A \text{ has reacted with } B
\end{cases}
\]

\[
W_A^{\text{out}} = p_A W_B^{\text{in}} + (1 - p_A) 0
\] (1)

\[
W_A^{\text{out}} = p_A W_B^{\text{in}}
\] (2)

\[
W_B^{\text{out}} = p_B W_A^{\text{in}} + (1 - p_B) 0
\] (3)

\[
W_B^{\text{out}} = r p_A W_A^{\text{in}}
\] (4)

Conversion based on lysine (\( p_B \)) can be related to the conversion based on glutamine (\( p_A \)) as follows:

\[
p_A = p \text{ and } p_B = \frac{9}{15} = 0.6 p_A
\] (5)

Using Figure 3 and the nomenclature of (17), we now define the following entities.

\[
\begin{align*}
1. & \quad E(W_A^{\text{in}}) = \text{Expected wt. of a molecule looking from } A \text{ inwards or towards the inside} \\
2. & \quad E(W_A^{\text{out}}) = \text{Expected wt. of a molecule looking from } A \text{ outwards or towards the outside} \\
3. & \quad E(W_B^{\text{in}}) = \text{Expected wt. of a molecule looking from } B \text{ inwards} \\
4. & \quad E(W_B^{\text{out}}) = \text{Expected wt. of a molecule looking from } B \text{ outwards}
\end{align*}
\]

We thus have:

\[
E(W_A^{\text{out}}) = p E(W_B^{\text{in}})
\] (6)

The expected weight of a molecule looking from \( B \) towards the inside is equal to its backbone weight plus the expected weight protruding out from its branches.

\[
E(W_B^{\text{in}}) = M + (g - 1) E(W_B^{\text{out}}) + f E(W_A^{\text{out}})
\] (7)

\[
E(W_B^{\text{in}}) = M + 14 E(W_B^{\text{out}}) + 9 E(W_A^{\text{out}})
\] (8)
Similarly, the expected weight of a molecule looking from A towards the inside is equal to its backbone weight plus the expected weight protruding out from its branches.

\[ E(W_A^{in}) = M + (f-1)E(W_A^{out}) + gE(W_B^{out}) \] (10)

However, since A and B groups are on the same molecule, we can write:

\[ E(W_A) = E(W_B) \] (11)

Therefore, the total expected weight of a molecule:

\[ E(W_A) = E(W_A^{in}) + E(W_A^{out}) \] (12)
\[ E(W_B) = E(W_B^{in}) + E(W_B^{out}) \] (13)

Now, what we need to do is to eliminate \( E(W_A^{out}) \) and \( E(W_B^{out}) \) from equation 10 and 12 to get the expected weight in terms of conversion \( p \), reactive group numbers \( f \) and \( g \) and \( r \).

From equation (10), (12) and (6), we get

\[ E(W_A) = M + fE(W_A^{out}) + gE(W_B^{out}) \] (14)
\[ E(W_A^{out}) = pE(W_B^{in}) \] (15)

Substituting for \( E(W_B^{in}) \) from equation 7 yields:

\[ E(W_A^{out})[1 - pf] = p \left( M + (g-1)E(W_B^{out}) \right) \] (16)
\[ E(W_B^{out}) = rp \left( M + (f-1)E(W_A^{out}) + gE(W_B^{out}) \right) \] (17)
\[ E(W_A^{out})[1 - pf] = p \left[ M + \frac{(g-1)rp(M + (f-1)E(W_A^{out}))}{1 - rpg} \right] \] (18)
\[ E(W_A^{out})[1 - pf] = pM + \frac{p^2(g-1)rM + p^2(g-1)(f-1)E(W_A^{out})}{1 - rpg} \] (19)
\[
E(W_A^{out}) \left(1 - rp\right) - \frac{p^2(g - 1)r(f - 1)}{1 - rpg} = pM + \frac{p^2(g - 1)rM}{1 - rpg} \tag{20}
\]

\[
E(W_A^{out}) = \frac{pM + \frac{p^2(g - 1)rM}{1 - rpg}}{(1 - rp) - \frac{p^2(g - 1)r(f - 1)}{1 - rpg}} \tag{21}
\]

\[
E(W_B^{out}) = \frac{rp\left(M + (f - 1)\right)}{1 - rpg} + \frac{pM + \frac{p^2(g - 1)rM}{1 - rpg}}{(1 - rp) - \frac{p^2(g - 1)r(f - 1)}{1 - rpg}} \tag{22}
\]

But \(E(W_A) = M + fE(W_A^{out}) + gE(W_B^{out})\), therefore:

\[
E(W_A) = M + f \frac{pM + \frac{p^2(g - 1)rM}{1 - rpg}}{(1 - rp) - \frac{p^2(g - 1)r(f - 1)}{1 - rpg}} + g\frac{rp\left(M + (f - 1)\right)}{1 - rpg} + \frac{pM + \frac{p^2(g - 1)rM}{1 - rpg}}{(1 - rp) - \frac{p^2(g - 1)r(f - 1)}{1 - rpg}} \tag{23}
\]

Equation 23 represents the relation between the expected weight average molecular weight \((M_W)\) of the polymer molecule as function of glutamine conversion and is illustrated graphically in Figure 4. You ought to say something about the predicted \(M_W\) in Figure 4. We need to emphasize here that this equation is based assuming no
intramolecular crosslinks. If intramolecular crosslinks are to occur, one would expect a slower increase in molecular weight.

From equation (23) we can calculate the conversion at gel point (infinite molecular weight) by equating the dominator to zero, this yields:

\[
(1 - rp) = \frac{p^2(g - 1)r(f - 1)}{1 - rpg}
\]  

\[
rp^2(f + g - 1) - p(f + rg) + 1 = 0
\]  

\[
p_{gel} = \frac{(f + rg) \pm \sqrt{(f + rg)^2 - 4r(f + g + 1)}}{2r(f + g - 1)}
\]

Equation 26 gives the conversion at the gel point for a general case. By substituting \( f = 9, g = 15 \) and \( r = 0.6 \), we find this conversion to be 0.058.

8.4 CONCLUSIONS

A polymerization model based on probabilistic approach has been developed for the crosslinking of \( \beta \)-lactoglobulin by transglutaminase enzyme. According to the model, at glutamine conversion of 5.8 will cause onset of gelation. Verification of model validity may be studied using combination of gel permeation chromatography with static light scattering, to calculate the absolute molecular weight and reverse phase high performance liquid chromatography to determine the glutamine conversion. The latter can be done via exhaustive proteolysis of the crosslinked protein by digestive enzyme as discussed elsewhere (19).
8.5 REFERENCES


Figure 1. Schematic representation of the reaction of glutamine and lysine residues in β-LG molecules to yield ε-(γ-glutamyl)lysine bonds
Figure 2. Amino acid sequence of β-LG (variant A) showing glutamine and lysine residues (Q and K, respectively)
Figure 3. Schematic representation of protein chains showing glutamine (Q) and lysine (K) residues showing the directions of $E(W_A^{in})$, $E(W_A^{out})$, $E(W_B^{in})$ and $E(W_B^{out})$. Directions 1 and 2 indicate the weight attached to a glutamine residue looking inwards and outwards, respectively. Directions 3 and 4 indicate the weight attached to a lysine residue looking inwards and outwards, respectively.
Figure 4. Molecular weight of β-LG polymers as a function of glutamine conversion, based on equation (23)
CHAPTER 9

MODULATION OF HYDROPHOBIC INTERACTIONS IN
DENATURED WHEY PROTEINS BY TRANSGLUTAMINASE
ENZYME

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the *Food Hydrocolloids* Journal.
Modulation of Hydrophobic Interactions in Denatured Whey Proteins

by Transglutaminase Enzyme

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ABSTRACT

The role of enzyme crosslinking in mediating formation of hydrophobic association in chemically denatured whey protein isolates (WPI) is examined. WPI samples denatured with dithiothreitol (DTT) and incubated at 50 °C with and without transglutaminase enzyme show dramatic differences in viscosity, with the viscosity of the sample exposed to enzyme less by several orders of magnitude than the sample without enzyme. Upon further exposure of both samples to sodium dodecyl sulfate (SDS) to eliminate hydrophobic associations, we observe no change in the viscosity of the sample previously treated with enzyme, suggesting this sample to have minimal hydrophobic associations. In contrast, the sample without enzyme shows a dramatic drop in viscosity indicating it to have had substantial hydrophobic associations. A similar trend but to a lesser extent is observed at a higher WPI concentration. These results taken together suggest that the formation of enzyme catalyzed ε-(γ-glutamyl)lysine bonds attenuates hydrophobic interactions through steric hindrance and formation of compact molecules that limits exposure of the hydrophobic moieties.

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9.1 INTRODUCTION

Whey proteins are widely used food ingredients as they can bestow multiple functionalities (1-3) such as emulsification, gelation, water binding, solubility, whipping/foaming and thickening, to food products. These functional attributes of whey proteins are dictated by the various chemical and physical bonds that take place among the protein molecules. Chemical bonds typically involve formation of disulphide (4-8) and enzyme-catalyzed ε(γ-glutamyl)lysine linkages (9-13) while physical bonds occur through hydrophobic, electrostatic and hydrogen bonding interactions. Chemical bonds are strong (200-400 kJ/mol) and permanent, whereas the physical interactions are transient and weaker (5-10 kJ/mol for hydrophobic interactions, 10-40 kJ/mol for hydrogen bonding, and 25-80 kJ/mol for electrostatic interactions) (14,15). Nevertheless, hydrophobic interactions in general are known to be an important factor in the organization of the constituent molecules of living matter into complex structural entities (16). Although the exact role of hydrophobic interactions in protein folding is not fully deciphered (17-19), there is no doubt that the conformations of the protein molecules affect and are affected by the extent of the hydrophobic interactions.

In this short note, we address the effect of enzymatic crosslinking of whey proteins by transglutaminase on the extent of hydrophobic interactions under denatured conditions. Protein molecules are denatured by dithiothreitol (DTT) and the rheological properties are monitored in the absence and presence of transglutaminase. The results are intriguing as they seemingly contradict earlier results reports but can be easily embraced in terms of changes in hydrophobic interactions caused by enzyme crosslinking.
9.2 MATERIALS AND METHODS

9.2.1 MATERIALS

Whey protein isolate (WPI) were obtained from Davisco Food International, (LeSueur, Mn). A commercial version of transglutaminase enzyme (1% Enzyme and 99% Maltodextrin, by weight) was supplied by Ajinomoto Co., Japan. Sodium azide, dithiothreitol (DTT) and sodium dodecyl sulfate (SDS) were obtained from Sigma Chemical Co. (St. Louis, MO). De-ionized water (>15MΩ) was used in all the experiments.

9.2.2 PREPARATION OF PROTEIN SOLUTION

WPI powder was dissolved in de-ionized water to the desired final concentration with no pH adjustment (pH ~ 6.9) and stirred for complete solubility. Sodium azide (2% by wt.) was added to prevent bacterial growth. DTT was then added (20 mM) and stirred. For the samples treated with enzyme, the enzyme was added in a concentration of (10 U/gm). All samples were incubated at 50 °C.

9.2.3 RHEOLOGICAL MEASUREMENTS

Rheological measurements were carried out using a Dynamic Stress Rheometer (DSR) (TA Instruments, New Castle, DE). A parallel plate geometry with 40 mm diameter plates and 1mm gap was used for the steady shear experiments whereas a standard couette geometry was used for the dynamic experiments.

9.3 RESULTS AND DISCUSSION

WPI samples (8%) containing 20 mM DTT doped either with or without transglutaminase enzyme were heated at 50 °C for 5 hrs. Comparison of these two types of samples in Figure 1 shows that the steady shear viscosity of the enzyme-treated is
three orders of magnitude lower than that of sample containing no enzyme. This is rather interesting as earlier work by us (10,20,21) showed that thermally denatured whey proteins, more specifically β-lactoglobulin, could be crosslinked using transglutaminase into high molecular weight aggregates or polymers having higher viscosity. However, in this case, enzymatic treatment in the presence of DTT which cleaves disulfide bonds causes the viscosity to be drastically lower compared with the sample with no enzyme. This suggests that the presence of enzyme has a considerable effect on the physical interactions between protein molecules. To examine the effect of hydrophobic interactions, we added 2% SDS to the previous samples. The viscosity of the sample containing no enzyme decreased drastically by several orders of magnitude upon addition of SDS (Figure 1). On the other hand, the sample with enzyme showed little change in viscosity, indicating that the enzyme-treated sample had minimal hydrophobic associations to begin with. The significance of these results can be explained as follows. Upon addition of DTT, the protein molecules are denatured and considerable hydrophobic associations take place. The fact that we have this scenario is validated by the fact that disruption of hydrophobic association by addition of SDS reduces the sample viscosity by over four orders of magnitude. On the other hand, the presence of enzyme (in the DTT denatured sample), causes reaction between glutamine and lysine residues creating ε-(γ-glutamyl)lysine bonds. The significantly lower viscosity of this sample, (Figure 1) compared to its counterpart without enzyme suggests that the hydrodynamic volume swept by hydrophobically associated protein molecules is much higher than that of the molecules crosslinked by enzyme. The fact that further addition of SDS to the enzyme-containing sample has no effect on viscosity, indicates that the enzyme
crosslinks effectively limits formation of hydrophobic associations. In this regard, Tanimoto and Kinsella (22) has shown that \(\beta\)-lactoglobulin crosslinked by transglutaminase in the presence of 10 mM DTT contains intramolecular bonds that impedes unfolding of the molecules upon heating, hence suggesting a compact nature of the polymerized \(\beta\)-lactoglobulin molecules leading to a smaller hydrodynamic volume. We may also expect the compact protein structures to hide the hydrophobic residues in the interior part of the aggregates and prevent hydrophobic associations. Further, possible branching caused by enzyme crosslinking may also sterically hinder the hydrophobic associations to form.

The present finding may look seemingly contradictory to previous work that showed an increase in viscosity with enzymatic treatment in the presence of DTT (9,12,13). However, these authors conducted the enzymatic treatment at 40 °C, which is less favorable to hydrophobic interactions than 50 °C, as the hydrophobic interaction are strengthened at elevated temperatures (23). Dickinson and Yamamoto (11) showed that enzymatic crosslinking resulted in gel formation at 13% and 14% protein, with elastic modulus (\(G'\)) in the order of \(10^3\) Pa, while samples without enzyme showed no gelation; however, the authors did not use DTT with the sample without enzyme.

We find that trends similar to Figure 1 are also observed when 10% whey protein solution is heated in the presence of DTT. This solution undergoes gelation upon incubating at 50 °C. Figure 2, which shows results of \(in situ\) experiments, reveal that the gel elastic modulus (\(G'\)) for the enzyme treated sample is lower than that without enzyme treatment. However, the difference in the values of \(G'\) is not as substantial as the difference in viscosity observed for the sample at 8% concentration. This is possibly due
to the involvement of other types of interactions such as hydrogen bonding and/or the entanglements of the protein chains. The significance of the results indicates that concentration of protein affects the extent of modulation of hydrophobic interactions by transglutaminase enzyme.

9.4 CONCLUSIONS

This study revealed that the presence of transglutaminase catalyzed $\varepsilon$-(\(\gamma\)-glutamyl) lysine bonds in WPI denatured using DTT impedes hydrophobic association formation. This was validated from comparison of viscosity data of 8% WPI containing DTT with and without enzymes. In the absence of enzymes, highly shear thinning viscosity was obtained versus Newtonian viscosity three decades lower in magnitude in the presence of enzyme. Upon SDS addition, no change in viscosity occurred in the presence of enzyme, indicating presence of minimal hydrophobic associations with enzyme treatment. In contrast, SDS addition to samples containing no enzyme dropped viscosity drastically, revealing the presence of dominant hydrophobic interactions in the absence of enzyme. These results can be explained on the basis that use of DTT cleaves disulfide linkages enabling formation of significant hydrophobic associations. Presence of enzyme produces crosslinks that prevents exposure of the hydrophobic moieties through steric hindrance and creation of compact molecules. A similar trend was noticed in the dynamic properties at a higher concentration (10%) where gels were formed, but to lesser extent possibly due to the involvement of other modes of interactions.
ACKNOWLEDGMENTS

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[22] Tanimoto, S. Y.; Kinsella, J. E. Enzymatic modification of proteins: Effect of 
transglutaminase cross-linking on some physical properties of β-lactoglobulin. 

Figure 1 Steady shear viscosities of 8% whey protein isolate samples (20 mM DTT) in the absence and presence of transglutaminase enzyme after incubation at 50°C for 5 hrs. Measurements were taken at 25 °C.
Figure 2 Evolution of elastic ($G'$) and viscous ($G''$) moduli of 10% whey protein isolate samples (20 mM DTT) in the absence and presence of transglutaminase enzyme while in situ incubation at 50°C. Measurements were taken in the linear viscoelastic regime at a frequency of 1 rad/s.
CHAPTER 10

CONCLUSIONS AND RECOMMENDATIONS
10.1 CONCLUSIONS

In this chapter, we briefly summarize the key aspects of this dissertation. In the preceding chapters, we have shown that crosslinking of whey proteins by transglutaminase enhanced the rheological properties of the gels at pH 4. We investigated the role of the physical and chemical interactions on the rheology of the gels. We also characterized the conformational characteristics that facilitate crosslinking of β-lactoglobulin by transglutaminase and investigated the characteristics of the resulting crosslinked chains using Fourier Transform Infrared (FTIR) spectroscopy. Moreover, we provided an approach to modulate the hydrophobic interactions in chemically denatured whey proteins through using transglutaminase enzyme. Some of the major findings of this study are summarized below.

- Enzymatic polymerization at pH 8 proved to be a viable route for creating ε-(γ-glutamyl)lysine bonds. Upon acidification of the polymerized whey proteins using GDL under cold-set conditions, gels (pH ~ 4) exhibited higher fracture/yield stress and strain compared to cold-set gels with no enzyme and conventional heat set gels at 80 °C.

- An alternative protocol for crosslinking has proved to be even more effective, in which a preheating step (80 °C for 1 hr) at neutral pH is conducted, followed by subsequent enzyme treatment and final cold-set gelation through acidulation with GDL. The disulfide and ε-(γ-glutamyl)lysine bonds formed through heat and enzyme treatment respectively produced polymerized whey protein solutions. The molecular weight of these samples increased with protein concentration with the role of additional enzyme crosslinking.
manifesting as higher molecular weights only at higher protein concentration. This seemed to suggest that enzyme crosslinking was predominantly intramolecular at lower protein concentrations. During acidification in the absence of enzyme, the gel modulus (G’) showed a maximum as the pH approached the isolectric point and then decreased considerably afterwards. However, the presence of \(\varepsilon-(\gamma\text{-glutamyl})\text{lysine bonds}\) retarded the modulus decrease and made the network more stable against electrostatic repulsion. Moreover, the fracture strain and stress revealed significant increase with enzyme treatment, but without affecting the microstructure in the length scales probed using confocal microscopy.

- Chemical bonding plays a dominant role in building the whey protein polymer with minor participation of the hydrophobic interactions and hydrogen bonding. With respect to cold-set gels, gelation is mainly dependent on the hydrophobic interactions and hydrogen bonding. The absence of hydrophobic interactions caused extensive syneresis and macroscopic phase separation, while gelation was completely prohibited by hydrogen bonding dissociation using 8 M urea. The complete absence of chemical bonds did not prevent gelation and had a mild effect on the elastic modulus of the gel. However, chemical bonding profoundly affected the gel strength and flexibility (i.e. critical or fracture strain). Gels with no disulfide bonding fractured at much smaller strain values compared with gels with disulfide bonds. The introduction of \(\varepsilon-(\gamma\text{-glutamyl})\text{lysine bonds}\) for samples with no disulfide bonds restored the strength and flexibility of the gels.
FTIR analysis revealed that thermal denaturation proceeds quite differently from chemical denaturation, as manifested in the spectra of the amide I region. Denaturation by chemical denaturants, dithiothreitol or β-mercaptoethanol, showed no effect on the α-helix but shifted the monomer to dimer equilibrium towards a higher monomer concentration, whereas denaturation by thermal treatment dissociated the β-sheets in the native structure and led to formation of new intermolecular β-sheets. The amide I spectra of the crosslinked preheated protein were similar to the spectra before crosslinking unlike the crosslinking of chemically denature proteins that changed the structure of the α-helix and induced intermolecular β-sheets. On the other hand, the C-H stretching mode was found to be affected by crosslinking, suggesting some changes in the hydrophobic interactions due to enzymatic crosslinking.

A probabilistic polymerization model has been developed for the crosslinking of β-lactoglobulin by transglutaminase enzyme. According to the model, the onset of gelation occurs at 5.8% conversion of glutamine.

Transglutaminase enzyme is capable of modulating hydrophobic interactions in chemically denatured whey proteins. Upon heating at 50 °C, whey protein solution-chemically denatured by DTT- exhibited highly shear thinning viscosity versus Newtonian viscosity three decades lower in magnitude in the presence of enzyme. Upon dissociation of the hydrophobic interactions by SDS no change in viscosity occurred in the presence of enzyme, indicating minimal hydrophobic associations. In contrast, SDS addition to samples containing no enzyme caused drastic drop in viscosity, revealing the presence
of dominant hydrophobic interactions. This phenomenon is explained on the basis that use of DTT cleaves disulfide linkages enabling formation of significant hydrophobic associations. Presence of enzyme produces crosslinks that prevent exposure of the hydrophobic moieties through steric hindrance and creation of compact molecules. A similar trend was noticed in the dynamic properties at a higher concentration (10%) where gels were formed, but to lesser extent possibly due to the involvement of other modes of interactions.

10.2 RECOMMENDATIONS

Although we have shown enhanced rheological properties of the acidic whey protein gels upon using transglutaminase, we believe that this is just the starting point that needs to be completed by further basic and applied research covering the following areas.

- We have chosen pH 4 to be representative of the acidic environment, however; if we changed the pH to 3 or 2, we expect entirely different gel rheology and microstructure. As we move further from the isoelectric point, it would be important to understand the effect of transglutaminase in enhancing the rheological properties at these new conditions.

- Since the microstructure of the gels with and without enzyme treatment looks identical under the confocal microscope and slightly different under the scanning electron microscopy, it would be interesting to move one step beyond towards higher magnification and examine the samples under the transmission electron microscopy.
• Complementing the FTIR studies by nuclear magnetic resonance (NMR) to further probe the chains conformations will be of primary importance to envisage the crosslinking requirements as well as the induced changes after the crosslinking.

• The observation we found in chapter 9 concerning the effect of transglutaminase in modulating the hydrophobic interactions opens the door towards a more general question: could enzymes be a tool to control hydrophobic interactions in food systems? To the best of our knowledge, this issue has not been discussed before and it will be important to pursue studies along this line.

• Application of the scientific findings remains a real challenge. So, it will be inevitable to include the modified whey proteins into a real food product and run a tasting panel by trained personnel to examine its suitability.
APPENDIX A
ADDITIONAL DATA
Figure 1. SEM micrograph showing the structure of 7.5% whey protein isolate gel at pH4. Gel was obtained by preheated at 80 C for 1 hr followed by acidification using GDL to pH4.
Figure 2. SEM micrograph showing the structure of 7.5% whey protein isolate gel at pH4. Gel was obtained by preheated at 80 C for 1 hr followed by acidification using GDL to pH4.
Figure 3. SEM micrograph showing the structure of 7.5% enzymatically modified whey protein isolate gel at pH4. Gel was obtained by preheated at 80 C for 1 hr followed by enzymatic treatment then acidification using GDL to pH4.
Figure 4. SEM micrograph showing the structure of 7.5% enzymatically modified whey protein isolate gel at pH4. Gel was obtained by preheated at 80°C for 1 hr followed by enzymatic treatment then acidification using GDL to pH4.
Figure 5. SEM micrograph showing the structure of 5% whey protein isolate gel at pH4. Gel was obtained by preheated at 80 C for 1 hr followed by acidification using GDL to pH4.
Figure 6. SEM micrograph showing the structure of 5% whey protein isolate gel at pH4. Gel was obtained by preheated at 80 °C for 1 hr followed by acidification using GDL to pH4.
Figure 7. SEM micrograph showing the structure of 5% enzymatically modified whey protein isolate gel at pH4. Gel was obtained by preheated at 80 C for 1 hr followed by enzymatic treatment then acidification using GDL to pH4.
Figure 8. SEM micrograph showing the structure of 5% enzymatically modified whey protein isolate gel at pH4. Gel was obtained by preheated at 80 C for 1 hr followed by enzymatic treatment then acidification using GDL to pH4.
Figure 9. Fracture stresses and strains of cold set whey proteins gels at pH 4. Fracture was carried out by compression fracture using universal testing machine. All of the samples were preheated at 80 °C for 1 hr then acidified by GDL to pH4. In sample 1 no additions were added, sample 2 has 2% SDS added to the protein solution immediately before GDL addition, sample 3 has been enzymatically crosslinked, while sample 4 is similar to sample 3 but with 4% β-mercaptoethanol added. Samples with no enzyme treatment, with β-mercaptoethanol was fragile and weak that they were fractured when extracted from the glass tube.
Figure 10. Confocal microscopy micrograph showing the structure of 7% whey protein isolate gel at pH4. Gel was obtained by preheated at 80 C for 1 hr followed addition of 2%SDS then acidification using GDL to pH4.
Figure 11. Confocal microscopy micrograph showing the structure of 7% whey protein isolate gel at pH4. Gel was obtained by preheated at 80 °C for 1 hr followed addition of 100mM DTT then acidification using GDL to pH4.
Figure 12. Confocal microscopy micrograph showing the structure of 7% whey protein isolate gel at pH4. Gel was obtained by preheated at 80 C for 1 hr followed addition of 20% urea then acidification using GDL to pH4.
Figure 13. Confocal microscopy micrograph showing the structure of 7% enzymatically modified whey protein isolate gel at pH 4. Gel was obtained by preheated at 80 C for 1 hr followed addition of 2%SDS then acidification using GDL to pH4.
Figure 14. Confocal microscopy micrograph showing the structure of 7% enzymatically modified whey protein isolate gel at pH4. Gel was obtained by preheating at 80 °C for 1 hr followed addition of 100mM DTT then acidification using GDL to pH4.
Figure 15. Confocal microscopy micrograph showing the structure of 7% enzymatically modified whey protein isolate gel at pH4. Gel was obtained by preheating at 80 °C for 1 hr followed addition of 20% then acidification using GDL to pH4.
Figure 16. GPC Chromatograms of whey protein isolate samples, preheated at 80 °C for 1 hr then incubated with transglutaminase enzyme (10U/g) for different periods of time as shown in the figure. Samples were extensively denatured using 8M urea, 2% SDS and 100 mM DTT before being injected in the GPC.
Figure 17. GPC Chromatograms of whey protein isolate samples, denatured by 100 mM DTT then incubated with transglutaminase enzyme (10U/g) for different periods of time as shown in the figure. Samples were extensively denatured using 8M urea, 2%SDS and 100 mM DTT before being injected in the GPC.
APPENDIX B

PUBLICATIONS
Polymerization and Gelation of Whey Protein Isolates at Low pH Using Transglutaminase Enzyme

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Dynamic and steady shear rheology is used to examine the synthesis of low-pH (~4) whey protein gels obtained through a two-step process. The first step involves cross-linking of whey proteins at pH 8 and 50 °C using transglutaminase enzyme, while the second step entails cold-set acidification of the resulting solution using glucono-δ-lactone (GDL) acid. During the first step, the sample undergoes enzyme-catalyzed ε-(γ-glutamyl)lysine bond formation with a substantial increase in viscosity. Acidification in the second step using GDL acid leads to a rapid decrease in pH with a concomitant increase in the elastic (G') and viscous (G'') moduli and formation of a gelled network. We examine the large strain behavior of the gel samples using a relatively new approach that entails plotting the product of elastic modulus and strain (G'') as a function of increasing dynamic strain and looking for a maximum, which corresponds to the yield or fracture point. We find the enzyme-catalyzed gels to have significantly higher yield/fracture stress and strain compared to cold-set gels prepared without enzyme or conventional heat-set gels. In addition, the elastic modulus of the enzyme-catalyzed gel is also higher than its non-enzyme-treated counterpart. These results are discussed in terms of the gel microstructure and the role played by the enzyme-induced cross-links.

KEYWORDS: Rheology; gel; whey protein; enzyme; transglutaminase; yield stress; cold set

INTRODUCTION

Whey proteins are important food ingredients that are used in a number of food products that includes dairy, confectionary, and dessert items. A major coproduct of the cheese industry, whey protein production (dry) has been exceeding 1 000 000 lb annually in the United States in recent years (1). In addition to their nutritional values, incorporation of whey proteins in food products can promote emulsification, stabilization, foaming, and gelation properties. In this regard, whey protein gelation is of considerable interest because it can provide new food products with unique functional performance and favorable textural properties (2–4). Moreover, food gelling systems are potential alternatives in replacing fats in foods (5). Whey protein gels can substitute the role of fats in enhancing textural properties of foods. Such gels are most commonly produced using heat treatment (2,3,6–10), although other factors such as salt addition (11), acidification (12,13), and enzyme treatment (14–19) can cause gelation of whey proteins with or without heating. In many food applications, however, it is not desirable to heat the products to high temperatures to induce gelation, and it becomes advantageous to induce gelation at ambient or near ambient temperatures (20).

Whey protein gels, prepared by heating acidic whey protein solution (pH < 4.6), are weak and brittle (21), limiting their use in food products under such conditions. Such poor rheological properties occur mainly due to the absence of the strong disulfide bonds at these acidic conditions and pH-associated effects on the denaturation and aggregation reactions (21). However, disulfide bonds can presumably be introduced through a two-step procedure that involves creating chemical cross-links at neutral pH by heating the whey protein solution (step 1), followed by acidifying the polymerized protein solution to the required pH (step 2) (21).

A powerful and alternative approach for producing permanent bonds in whey proteins is by using enzymes (4,17,22,23). The specificity of enzyme-catalyzed cross-links combined with its ability to work at different (low) pH and low temperature makes this a viable route for developing whey protein gels. In this regard, earlier work in our laboratory focused on direct cross-linking of whey proteins at low pH. Burke et al. (23) treated whey proteins at pH 4 with transaminase using a combined enzyme and heat treatment protocol (incubation with enzyme at 40 °C for 40 min, followed by incubation at 80 °C for 20 min). The resulting gels exhibited higher elasticity and strength than that produced through conventional treatment. In the present study, we take a different approach to producing whey protein gels at low pH using transglutaminase enzyme. Transglutaminase (TG, EC 2.3.2.13), which has been used to cross-link whey protein (4,17,23–27), links glutamine and lysine residues as it catalyzes the acyl transfer reaction, and produces ε-(γ-glutamyl)lysine bonds. Although cross-linking by TG has been studied for some proteins such as αs1-casein, β-casein, β-casein, κ-casein, lyso and 7s soy globulins (28–36), cross-linking of whey proteins using TG is still not well developed. Most of the previous work used denaturant (such as dithiothreitol) to
denature the protein in order to facilitate cross-linking (15, 27-
37, 38). However, the use of such reagents is not allowed in
food applications. Moreover, the cold-set whey protein gelation
using TG has not been studied. In particular, the role of TG-
induced bonds in fortifying the whey protein network at low
pH has not been discussed.

In this study, we adopt a two-step strategy to develop whey
protein gels using enzymatic treatment at alkaline conditions
followed by cold setting using glucono-δ-lactone acid to low
pH (4). While a two-step procedure has previously been used
(21), it has been for heat-treated samples at high temperatures
and without any enzymes that can lead to new bond formation
and deterioration. The distinctive feature of our work involves
the combined use of enzyme (TG) with cold-setting conditions,
monitoring the initial enzyme-catalyzed polymerization step both
rheologically and biochemically, and evaluating the gelation
characteristics in-depth. In this regard, we use a new dynamic
rheological approach to explore the large strain behavior of
samples and identify yield/fracture points. We investigate the
different conditions affecting cross-linking by TG gels. Gels produced by enzymatic treatment at alkaline conditions
followed by cold setting using GDL acid to low pH were
compared to gels produced with no enzyme but cold set and
those prepared with conventional heat treatment. Our results
reveal enzymatic treatment to produce gels with superior
rheological properties. These results are discussed in terms of
the microstructure and role of enzyme-induced cross-links in
the gel network.

MATERIALS AND METHODS

Materials. Whey protein isolate (WPI) was obtained from Davisco
Food International (LeSueur, MN) and used as received. A commercial
version of transglutaminase enzyme (1% enzyme and 99% milkedextrin,
by weight) was provided by Ajinomoto Co., Japan. Enzyme activity
was determined using the hydrazinone method as described by Folk
(39). The activity unit is defined as the amount of enzyme producing
1 μmol of hydrazinone per minute at 37 °C. Glucono-δ-lactone
(GDL), N-ethylmaleimide, was purchased from Fisher Scientific
(Pittsburgh, PA). Sodium chloride (≥ 99%) was purchased from Sigma
Chemicals (St. Louis, MO). Deionized water (≥ 15 MΩ) was used in
all the experiments.

Sample Preparation. Protein solutions were prepared by dissolving
WPI powder in deionized water to obtain a final concentration of 7.5%
w/v and stirring for about 1 h to ensure complete solubility. The pH
was adjusted by using either NaOH (1 M) or HCl (1 and 0.1 M).
Solutions were degassed for about 15 min under vacuum of 25 in. Hg
at room temperature to eliminate trapped air bubbles.

Samples with enzyme were prepared by adding the requisite amount
of powdered enzyme to WPI solutions contained in vials and mixing
for 20 min at room temperature. The vials were closed immediately
after enzyme addition to prevent enzyme deactivation through air—
enzyme contact. Samples were then incubated for 5 h at 50 °C to induce
enzymatic reaction. Samples without enzyme were heated at 50 °C
for 5 h to mimic the thermal treatment of the samples with enzyme.
N-Ethylmaleimide (NEM) was added to some samples to prevent
disulfide bonding.

To study the effect of ionic strength on cross-linking of protein by
enzyme using NaCl was dissolved in the WPI solution at pH 8 before
enzymatic treatment. The molar concentrations of NaCl ranged from
0 to 200 mM.

WPI gels at pH 4 were obtained by adding 1.5% GDL to the WPI
solution (no salt added, originally at pH 8) at 25 °C and stirring
for about 10 min. The solution was then poured into a rheometer cup. All
rheological measurements were conducted at 25 °C, unless otherwise
mentioned. Conventional heat-treated gels were obtained by heating
WPI solution (7.5% w/w, pH 4) at 80 °C for 5 h.

Gel Electrophoresis. Polyacrylamide gel electrophoresis (PAGE)
under reducing conditions was performed on whey protein samples
using Mini-PROTEAN 3 Electrophoresis Cell (BIO-RAD, Hercules,
CA) to obtain information on their molecular weights. Protein solutions
(7.5%) were diluted 40 times (i.e., ratio of protein to buffer was 1:40)
by Laemmli sample buffer (25% glycerol, 2% SDS, and 0.01%)
Brucine blue R. mixed with 50 μL of β-mercaptoethanol/mL of
solution and heated for 5 min at 95 °C and then subjected to
electrophoresis in gradient gels of 4-15% polyacrylamide using the
discourricular system (40). These gels were stained with 0.1% Stain
(Stain Blue dye) and destained using first a solution of high destaining power
(40 wt % ethanol and 10 wt % acetic acid in deionized water) for 2 h
and later a solution of low destaining power (10 wt % ethanol and 10
wt % acetic acid in deionized water) for approximately 12 h. The gels
were subsequently immersed in a drying solution (containing 20%
ethanol and 10% glycerol in deionized water) for 30 min and fixed in
drying frames for 24 h. The dry gels were finally examined to determine
the different molecular weight bands. Gels under nonreducing condition
were prepared the same way as above but without the addition of
β-mercaptoethanol.

Rheological Measurements. Steady and dynamic shear rheological
experiments were conducted on a TA Instruments AR2000 rheometer
using a cone geometry (cup diameter = 30 mm, and bob diameter
= 28 mm). In the steady shear experiments samples were heated to 50
°C and maintained at this temperature. Viscosity was measured at 5-min
intervals while subjecting the sample to a continuous shear rate of 50
s⁻¹. In the dynamic experiments the samples were subjected to
a sinusoidal deformation as either a function of increasing stress amplitude
or frequency of oscillation, and the corresponding elastic (G') and
viscous (G'') moduli were measured. The strain sweep experiments,
which were conducted at a constant frequency of 1 rad/s, served two
purposes. First, it provided the limit of linear viscoelasticity (LVE)
that could be used in the frequency experiments. Second, as discussed
later, it provided a method to examine yield and fracture stress/strain
of the samples (41, 42). The frequency spectrum of the elastic and
viscous modulus, on the other hand, provided a signature of the state
(e.g., liquid or gel) of the samples (43). In all experiments conducted,
the samples were covered with n-hexadecane to prevent evaporation.

Field Emission Scanning Electron Microscopy. (FESEM). FESEM
(JEOL JEM 6400F) was used with an accelerating voltage of 5 kV and
a magnification of 10,000 × to investigate the microstructure of whey
protein gels obtained under different conditions. Small pieces of gels
were fixed with 2.5% (w/v) glutaraldehyde in water for 1 h and rinsed
with water 3 times. The samples were then soaked in 0.2% (w/w)
osmium tetroxide for 12 h. The gels were initially dehydrated using a
graded ethanol series (44) and completely dried using critical point
drying with CO₂ (45). The dried samples were gently fractured and
coated with Au/Pd by diode sputter coating prior to scanning.

RESULTS AND DISCUSSION

Enzyme Activity at Different pH. To develop the two-step
gelation process, it is essential to (i) determine the conditions
in which the enzyme will have sufficient activity and subse-
sequently (ii) examine which of these conditions are most
conducive for the enzyme-catalyzed polymerization of whey
protein. In this regard, we first explored the effect of pH on
enzyme activity at 50 °C. (Figure 1). We chose this temperature
as it had been found to be the optimal temperature for
transglutaminase use (Ajinomoto Data Sheet). From Figure 1,
which shows relative activity as a function of time, it is apparent
that enzyme activity decays rapidly for pH < 5 or > 8. This
behavior can be explained in terms of protonation and depro-
tonation of enzyme active site (cysteine) at low and high pH,
respectively (45), or due to disruption of the active site conformation resulting from enzyme denaturation. We also find
enzyme activity to be fairly stable for pH 5.5–7, and to have lower
degree of stability at pH 8. On the basis of these results, we
undertook enzymatic treatment of WPI samples at pH 6.7, 7, and
8, as detailed in the next section. Studies of enzymatic polymerization of WPI at pH 4 and 9 were not undertaken

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Figure 1. Effect of pH on the relative activity of transglutaminase enzyme at 50 °C.

because of the rapid decay of enzyme activity under these conditions. Enzymatic polymerization at pH 5 was also excluded because native (not subjected to any treatment) whey protein precipitates out of solution immediately after the solution pH is lowered to this value.

Whey Protein Polymerization with Enzyme. The effect of pH on enzyme treatment of whey proteins was examined by incubating samples at 50 °C for 5 h with 100 U of enzyme/g of protein at pH 6, 7, and 8. Figure 2 shows the molecular weight of various samples following enzyme treatment obtained using reducing SDS-PAGE analysis. In Figure 2a the two bands appearing on the lowest (bottom) part of the gel correspond to α-lactalbumin and β-lactoglobulin, as evident from the SDS-PAGE analysis of these individual components (Figure 2b). We find the β-lactoglobulin band to remain effectively unchanged upon enzyme treatment at pH 6 and 7 but to become quite faint at pH 8. On the other hand, the α-lactalbumin band disappears at all pH following enzyme treatment. These results indicate that α-lactalbumin can be readily cross-linked (polymerized) at different pH, consistent with earlier reports (15,46). However, β-lactoglobulin requires slightly alkaline medium to be cross-linked. The difference in behavior between the two proteins can be attributed to the molten globular state of α-lactalbumin that facilitates cross-linking at all pH due to the disordered tertiary structure (26). In contrast, β-lactoglobulin is believed to get partially denatured at a high pH of 8, which then facilitates its cross-linking by the enzyme (47). To study the individual behavior of α-lactalbumin and β-lactoglobulin, we incubated pure fractions of α-lactalbumin and β-lactoglobulin with enzyme (Figure 2b). We can clearly observe that at pH 8, β-lactoglobulin molecules are cross-linked with each other in the absence of α-lactalbumin (lane 9). We can also observe from the same figure that pure fractions of α-lactalbumin are able to polymerize irrespective of the pH (lanes 3, 4, and 5), whereas β-lactoglobulin needs alkaline pH to polymerize. On the basis of these results, pH 8 represents a viable environment for the cross-linking of whey proteins by transglutaminase. Any enzymatic treatment reported in the rest of this study has therefore been conducted at a pH of 8.

To examine the relative portion of enzyme versus disulfide linkages formed when whey proteins are treated at 50 °C for 5 h, SDS-PAGE analysis was undertaken for both reducing and nonreducing conditions. Figure 2c shows these results for whey protein samples (pH 8) subjected to various types of treatment. Lane 2, which corresponds to native WPI, reveals that there is almost no polymerization occurring. This indicates that although whey proteins are partially denatured, heat treatment is needed to initiate polymerization. Treatment of WPI at 80 °C for 1 h (lane 3) reveals extensive polymerization via disulfide bonding (note the effect of reducing conditions in decreasing the molecular weight in lane 4). Heating the sample at 50 °C for 5 h (lane 5) produces a weak high molecular weight band and some aggregates with molecular weight less than 93 kDa (i.e., tetramers and lower). In contrast, the presence of enzyme (lane 7) under similar conditions reveals a large proportion of high molecular weight aggregates that remain unabsorbed even under reducing conditions (pH 8). This suggests that disulfide bonds.
Whey Protein Isolates at Low pH

Figure 3. SDS–PAGE bands for enzymatic polymerization of whey protein isolate samples at different (a) enzyme and (b) NaCl concentrations. All experiments were conducted at pH 8. (a) Lane 1: molecular weight marker. Lane 2 is native whey protein isolate sample. Lanes 3–10 are whey protein isolate samples treated with enzyme at 50 °C for 5 h at different concentrations as follows: lane 3 [1 mg of protein], lane 4 [5 mg of protein], lane 5 [10 mg of protein], lane 6 [20 mg of protein], lane 7 [30 mg of protein], lane 8 [40 mg of protein], lane 9 [50 mg of protein], and lane 10 [100 mg of protein]. (b) Lane 1: molecular weight marker. Lane 2 is native whey protein isolate sample. Lane 3 is sample heated at 50 °C for 5 h without enzyme. Lanes 4–6 are samples treated with 10 mg of enzyme at 50 °C for 5 h: lane 4 (no salt added), lane 5 [20 mM NaCl], lane 6 [50 mM NaCl]. Lane 7 [100 mM NaCl], lane 6 [200 mM NaCl].

The effects of enzyme concentration and solution ionic strength on whey protein polymerization are displayed in Figure 3. SDS–PAGE analysis following incubation with different concentrations of enzyme at pH 8 and 50 °C for 5 h is shown in Figure 3a. We find no further effect on polymerization for enzyme concentrations higher than 10 mg/g of protein. This can be surmised from the constant intensity of the monomeric bands of α-lactalbumin and β-lactoglobulin from 10 to 100 units (lanes 5–10). Figure 3b shows the effect of salt concentration on the extent of polymerization. These samples (pH 8) were incubated with enzyme for 5 h at 50 °C. It is apparent that an increase in ionic strength decreases the extent of polymerization of whey protein chains. At larger salt concentrations, darker (or heavier) bands of α-lactalbumin and β-lactoglobulin, indicating a higher concentration of unpolymerized fraction of protein, is noticed. This behavior can be explained on the basis of electrostatic interactions. At higher ionic strength, these interactions between protein chains are screened and the chains collapse. This collapse prevents the "induced fit" required for enzyme action. According to the induced fit concept, the substrates (α-lactalbumin and β-lactoglobulin in our case) cause the three-dimensional structure of the enzyme to change. This change in the structure brings

![Figure 4](image-url)  

Figure 4. Apparent viscosity (at a shear rate of 50 s⁻¹) of whey protein isolate samples as a function of incubation time with enzyme. Results are shown for different pHs. The catalytic groups (glutamine and lysine) into proper alignment and induce the (c-(γ-glutamyl)lysine) bonds. The collapsed chains of the α-lactalbumin and β-lactoglobulin molecules at high ionic strength fail to produce the proper enzyme conformation.

To investigate the effect of the enzyme treatment on rheological properties, steady shear viscosity was measured at a fixed shear rate (50 s⁻¹) as a function of incubation time (Figure 4). No increase in viscosity is observed when incubating the enzyme with the whey protein solution at 50 °C at pH 6 or 7. This indicates that the few cross-links (mainly between α-lactalbumin molecules) have little effect on solution viscosity. On the other hand, there is an appreciable increase in viscosity at pH 8 due to the polymerization of both β-lactoglobulin and α-lactalbumin molecules, consistent with our SDS page analysis. It should be noted that heating whey protein at 50 °C and pH 8 induces some denaturation formation. This is also exhibited in Figure 4, in which we observe a modest increase in viscosity for a sample containing no enzyme.

Cold-Set Acidic Gels following Enzyme Treatment. After enzyme treatment at pH 8, the protein solution was acidified by GDL (1.85 wt %), a slow release food-grade acidulant, to pH 4. The use of GDL over other strong acids (e.g., HCl) thereby allowed the time scale of sample pH change to be long enough to produce homogeneous gelation throughout the sample. Please note that minimal, if any, enzyme cross-linking occurred during acidification as the enzyme had negligible activity (Figure 1) following the initial polymerization step. Figure 5a shows the change in protein pH following GDL addition. We observe two distinct regimes consisting of a large initial decrease in pH to approximately 4.7 in about 2 h followed by a much slower decrease to pH 4 spanning 48 h. In Figure 5b we examine the corresponding change in elastic (G') and viscous (G'') modulus upon GDL addition. For comparison purposes, results of a sample without any enzyme are also included. This sample was incubated at 50 °C for 5 h to mimic the procedure that the enzyme-treated samples received. Gelation of both samples was monitored in situ in the rheometer at a constant strain amplitude (0.1%) and frequency (1 rad/s).

Several features are apparent from Figure 5b. First, there is a substantial increase in moduli with acidification as repulsion between protein chains starts to diminish with a decrease in pH. Most of this increase occurs within the first 2 h, consistent with the initial pH decay to 4.7 observed in Figure 5a. Second, following the initial increase in moduli, we observe a slight decrease in their value with time. The maximum in moduli may
Figure 5. Changes in whey protein properties during cold-set acidification through addition of 1.85% glucono-δ-lactone (GDL) acid. The original sample was enzyme treated at pH 8. (a) Change in pH with time upon addition of GDL. Inset at the upper right corner is an enlarged view of the initial pH decrease with time. (b) Evolution of the elastic (\(G'\)) and viscous (\(G''\)) moduli of the enzyme-treated whey protein sample during decrease of its pH.

be attributed to the sample passing through the isoelectric point. Third, the elastic (as well as the viscous) modulus of the enzyme-treated sample is an order of magnitude higher than that of the sample containing no enzyme. Finally, the elastic modulus of both samples is larger than their corresponding viscous modulus, suggesting presence of a gelled network.

It is pertinent to verify the presence of this gelled network upon acidification, since the previous results were monitored at a single frequency. Figure 6 shows the frequency spectrum of the elastic and viscous modulus of both samples (circles corresponding to enzyme treated and squares without enzyme treatment) after 48 h of acidification. For both cases, we find \(G'\) and \(G''\) to exhibit a very weak frequency dependence, with \(G'\) larger than \(G''\) over the entire frequency range observed. Such features are characteristics of a gel. In addition, the sample treated with enzyme has a higher elastic modulus compared to the untreated sample. Since \(G'\) is related to the degree of cross-linking, this suggests the presence of additional cross-links in the enzyme-treated sample.

An important issue to consider when examining gel modulus is the extent of disulfide linkages formed upon heating the sample at 50 °C for 5 h and its impact on gel rheology. To examine this, samples were prepared in which N-ethylmaleimide (NEM) was added to the whey protein solution (pH 8) prior to heating at 50 °C for 5 h. NEM is believed to prevent disulfide bonding by blocking the free sulfhydryl groups. The samples were then cold set by GDL to pH 4. The frequency spectrum of the dynamic moduli of such a sample is shown in Figure 6 (triangles). We find both \(G'\) and \(G''\) of the samples with and without NEM to be essentially identical, thereby demonstrating several important points. First, the number of disulfide linkages formed during the polymerization step is few, consistent with our SDS–PAGE results (Figure 2c). Second, these disulfide linkages have a negligible effect on gel rheology, with physical interactions being the primary mechanism for gel formation (without enzyme). Finally, the enhanced modulus observed upon enzyme treatment can be directly attributed to the additional enzyme-catalyzed bonds formed.

Large Strain and Yield Behavior. Figure 7a shows the dynamic moduli of the enzyme-treated and untreated samples as a function of increasing strain amplitude. At low strains, both \(G'\) and \(G''\) are flat, reminiscent of materials in the linear
viscoelastic regime. With increasing strain, however, nonlinearity sets in with a slow decrease of both $G'$ and $G''$. Subsequently, $G'$ and $G''$ tend to cross over as the material microstructure gets disrupted. Eventually at large strains, $G''$ becomes larger than $G'$ and both decrease rapidly as the material yields and/or fractures. Qualitatively, we find the yield strain of the enzyme-treated sample to be larger than the untreated sample. However, it is difficult to pinpoint the yield (or critical strain) exactly. One approach to do this has been to draw asymptotes through the low and large strain values of $G'$ (as shown in Figure 7a) and call the intersection point the yield point (41, 42). However, such an approach can produce errors because of the variation possible in drawing the asymptotic lines. It is interesting to note that reversible “heating” of sample microstructure is possible in the immediate vicinity of the yield strain regime (data not shown) if the material is left to relax.

An alternative and precise way to determine fracture/yield strain or stress is to plot the elastic stress, defined as a product of the elastic modulus and strain ($G\gamma$), as a function of increasing strain (41, 42, 48). Such an approach has been used effectively for particular (41) and polyacrylamide (42) gels to locate the yield point, which corresponds to the maximum in the plot. Figure 7b shows the elastic stress as function of increasing strain for enzyme-treated and untreated samples. In both cases, we observe a sharp maximum that corresponds to the yield/fracture stress and strain. In our samples, this point possibly corresponds to fracture rather than yield as samples beyond this strain showed no reversibility (data not shown). In fact, a fracture surface was visually identified in the rheometer geometry at/beyond the point of maximum stress. In addition, the strain values corresponding to the maximum stress in this figure are higher than those obtained from the yield point in Figure 7a.

We find from Figure 7b that the fracture stress of the enzyme-treated sample is about an order of magnitude higher than the untreated sample whereas the fracture strain is larger by about 50%. The same result is also obtained if we plot the total stress (instead of the elastic stress) as a function of strain (data not shown). This is because the dominant component of the total stress is the elastic stress. Table 1 summarizes the values for the yield and fracture stresses and strains. The higher values of the yield/fracture strain and stress, as well as the elastic modulus, in the case of enzymatic treatment can be attributed to the presence of chemical cross-links (ε-γ-glutamyl)lysine bonds) that strengthen the network. In contrast, the absence of such chemical cross-links in the untreated sample causes the gel network to yield/fail at lower strain and stress values.

**Cold-Set, Enzyme-Treated versus Conventional Heat-Treated Gels.** Protein gels are commonly prepared through heat treatment, typically at 80 °C. This produces complete heat denaturation of protein molecules, leading to hydrophobic, hydrogen, and electrostatic interactions together with disulfide bond formation (49–51). Conventional gels at pH 4 were obtained by heating whey protein solution (7.5% w/w, pH 4) at 80 °C for specified time intervals. Figure 8 compares the frequency spectra (at 25 °C) of both conventional heat-set gels and an enzyme-treated cold-set gel. In this regard, heat-set gels obtained using two different heating times, 1 and 5 h, are shown. We find conventional gels obtained after 1 h of heating to have a lower $G'$ than the enzyme-treated gels, whereas that heated for 5 h exhibits a slightly higher $G'$ than the enzyme-treated sample. The latter can be a result of the denaturation of most of the protein chains in the conventional gels, which are strengthened by physical interactions with prolonged heating, whereas only part of the protein chains in the cold-set gel denatures. The higher degree of denaturation allows more protein chains to be part of the network forming the gel, thereby leading to a larger modulus. In fact, the inset in Figure 8 shows the evolution of $G'$ of conventional gels (at 80 °C, $\omega = 1$ rad/s) as a function of heating time for the conventional gel.
respectively. It is interesting to note that although the conventional gel with 5 h of heat treatment has a higher elastic modulus, its yield and fracture properties are much lower in value compared to the enzyme-treated gel. These results highlight the fact that the elastic modulus alone is insufficient to characterize whey protein gels without knowledge of its yield and fracture properties. Interestingly, yield properties are of special importance in food applications as they mimic the chewing and biting of food.

Figure 10 shows SEM images of the microstructures of whey protein gels obtained under different conditions, conventional (5 h), cold set without enzyme treatment, and cold set with enzyme treatment. The conventional gel shows large voids and a nonhomogeneous particulate structure. These large voids in the structure may be the reason for the lower values of fracture stress and strain. This is because fracture occurs at the weakest parts of the gel network, which are expected to be the large void regions. On the other hand, both cold-set gels (with and without enzyme treatment) reveal a more homogeneous network structure. Although the cold-set gel without enzyme treatment appears more uniform and homogeneous than its enzyme-treated counterpart, it has lower fracture properties. This can be attributed to the presence of additional chemical cross-links (γ-glutamyl)lysine bonds) in the enzyme-treated gel, making it capable of carrying larger stresses with higher reversible extensibility (rubberiness).

CONCLUSIONS

In this study, we examined the rheological characteristics of a low-pH whey protein gel prepared using a new enzymecatalyzed two-step process. The first step involved enzymatic polymerization at pH 8 and 50 °C using transglutaminase. The second step entailed formation of gels at pH 4 through acidification of the polymerized whey using GDL under cold-set conditions. The alkaline conditions for the first step were chosen based on the relative activity of enzyme under different pH and its ability to polymerize β-lactoglobulin, the major component of whey protein, at this pH. Enzyme catalysis during this process led to the formation of γ-glutamyl)lysine bonds and a substantial increase in molecular weight and sample viscosity. The low-pH gels obtained through the second acidification step exhibited substantially higher fracture/yield stress and strain compared to the cold-set gel without enzyme treatment.
comparing to cold-set gels with no enzyme and conventional heat-set gels at 80 °C. In this regard, we used a new approach to obtain yield/fracture properties from dynamic rheological experiments. The elastic moduli of the enzyme-catalyzed gel was also higher than the one without enzyme. Interestingly, the modulus of the heat-treated sample was a function of heating time and lower than the enzyme-treated sample except for samples heated for a prolonged period (>5 h). Even these gels had comparable or slightly higher modulus than the enzyme-treated sample but considerably lower fracture stress/strain. These results taken together indicate the use of transglutaminase enzyme as a viable approach to produce low-pH gels with enhanced rheological characteristics.

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Enzyme-assisted Protein Gels: Rheology and Microstructure

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ABSTRACT

Enzymes offer a powerful tool to tailor the rheology and functional characteristics of food biopolymers. We use such an approach to develop and investigate novel enzyme-catalyzed whey protein gels at low pH (≈4). We adopt a multistep approach for gel formation: the proteins are initially polymerized using heat treatment and enzyme catalysis, and then acidified by slow release acidulant to obtain the gel. The properties of this gel are compared with conventional gels formed (without enzyme) under similar conditions. Enzyme-treated samples reveal modestly higher values of the elastic modulus (G') but substantially higher yield/ fracture strain compared with gels without enzyme treatment. The enzyme-facilitated gels exhibit gradual disruption of network structure with increasing strain in contrast to abrupt fracture observed for conventional gels. Interestingly, micrographs obtained using SEM reveal both gels to have similar microstructures thereby indicating no direct relationship between gel fracture properties and microstructure.

INTRODUCTION

Whey proteins are important ingredients in a number of food products such as dairy, confectionary, and desserts. Gelation of whey protein is of considerable interest because it provides unique functional performance and favorable textural properties [1]. Whey protein gels are typically produced in a single step by heat treatment (denaturation followed by polymerization) although a two-step process, involving heat treatment at low ionic strength, and/or far from isoelectric point (pI), followed by increasing ionic strength and/or adjusting the pH, to screen the repulsive forces and cause gelation [2, 3] have sometimes been used. However, whey protein gels at acidic conditions (pH < 4.6) are weak and brittle [4], limiting their use in food products. This is mainly due to the absence of the strong disulphide bonds at such acidic conditions [4]. Enzyme treatment offers a powerful and viable method to introduce such bonds (or crosslinks) in the polymerization step [5-8]. Transglutaminase (TG, EC2.3.2.13) has been used to crosslink whey proteins [6, 7, 9, 10] by crosslinking glutamine and lysine residues, as it catalyses the acyl transfer reaction and produces ε-(γ-glutamyl)lysine bonds. In this work, we examine a multistep approach of formulating whey protein gels by preheating the whey proteins (pH 7.0) at 80 °C for 1 hr, followed by enzyme treatment at 50 °C for 10 hrs, and then slow acidification to pH 4 by adding glucono-δ-lactone (GDL) acid.

MATERIALS AND METHODS

Whey protein isolate (WPI) was obtained from Davisco Food International, LeSueur, MN. Transglutaminase enzyme was provided by Ajinomoto Co., Japan. Enzyme was added as 10 U/g protein in all the samples. Sodium azide and glucono-δ-lactone (GDL) was obtained from Sigma Chemical Co. St. Louis, MO. De-ionized water was used in all the experiments (>15MΩ). Gel Permeation Chromatography (GPC) was done using the Waters 2690 separation module connected to multi angle light scattering detector, photo array detector and refractometer. Rheological measurements were carried out in a TA (previously know as Rheometric Scientific) ARES strain controlled rheometer. Couette geometry was used in all the experiments. For, scanning electron microscopy (SEM), the samples were initially dehydrated by a graded ethanol series and then critical point dried with CO₂. Dried samples were gently fractured and coated with Au/Pd by diode sputter coating prior to scanning.
RESULTS AND DISCUSSIONS

The first step in the gel formation procedure is to heat the whey proteins. Whey proteins denature upon heating and form high molecular weight aggregates. Figure 1 shows the chromatograms of native (original and untreated), preheated and enzyme treated whey protein samples.

![Figure 1. Chromatograms of native, preheated and enzyme treated whey proteins.](image)

The chromatogram of the native protein shows three peaks, 1, 2 and 3 corresponding to Bovine Serum Albumin (BSA), β-lactoglobulin (β-lg), and α-lactalbumin (α-lac), respectively. After preheating at 80 °C for 1 hr at 5% and 7.5%, the chromatograms show formation of aggregates (polymers). It should be noted that preheating is a pre-requisite to enzyme crosslinking as it unfolds the proteins needed for enzyme action. Upon treatment with enzyme, we observe further increase in molecular weight due to crosslinking. We also notice broadening and splitting of the aggregate peaks (at 7.5%). The weight average molecular weight ($M_w$) as well as the polydispersity index (PDI) for the different samples are shown in Table 1.

### Table 1. Molecular weight and poly dispersity index of protein aggregates

<table>
<thead>
<tr>
<th>Sample</th>
<th>$M_w$ (10^6 Da)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% Preheated</td>
<td>2.76 ± 0.08</td>
<td>1.52 ± 0.01</td>
</tr>
<tr>
<td>5% Enzyme Treated</td>
<td>2.85 ± 0.06</td>
<td>1.56 ± 0.01</td>
</tr>
<tr>
<td>7.5% Preheated</td>
<td>7.20 ± 0.20</td>
<td>1.9 ± 0.01</td>
</tr>
<tr>
<td>7.5% Enzyme Treated</td>
<td>10.05 ± 0.31</td>
<td>2.1 ± 0.03</td>
</tr>
</tbody>
</table>

After polymerization, samples were slowly acidified by GDL, and left to set at room temperature (25 °C) for 24 hrs. The amount of GDL needed was found to be linearly dependant on protein concentration (data not shown). Figure 2 shows the evolution of the elastic ($G'$) and viscous ($G''$) moduli with time for the 5% sample with and without enzyme treatment. Higher values of elastic modulus are obtained upon enzymatic treatment. It is interesting to note that $G'$ shows a maximum after about 4 hrs. Approximately at this time, the pH of the sample is ~5, which is the pl of β-lactoglobulin, the main component of whey proteins. At this pH, the repulsion between protein chains is minimal. After ~4 hrs, $G'$ decreases due to further decrease in pH, which creates a net positive charge on the protein molecules, promoting repulsion. This repulsion is thought to cause some protein chains to escape the gel network. We can clearly notice that the decrease in $G'$ in the case of enzyme treated gel is much less than in the gel with no enzyme. We believe that the presence of ε-(ɣ-glutamyl)lysine bonds limits the number of protein chains escaping the gel network.

![Figure 2. Evolution of $G'$, $G''$ after GDL addition](image)

The frequency spectra of the dynamic moduli following acidification, is shown in Figure 3. We observe a gel network for both cases (enzyme treated and conventional). In fact, the mechanical spectra of both gels look similar, with a scaling of $G'\sim \omega^{0.1}$. The only difference seems to be the modestly higher $G'$ of the enzyme treated sample. The similarity in behavior is suggestive that the gel microstructure or gel formation mechanism is analogous in the two cases. However, enzyme treated gels fracture at larger values of strains (i.e., become more deformable) as compared with gels without enzyme treatment (Figure 4). In this figure, the fracture point is identified as the maximum of elastic stress ($G'\gamma$). This enhanced deformability is believed to be due to the additional chemical crosslinks - ε-(ɣ-glutamyl)lysine bonds - created by the enzyme. These additional crosslinks not only affect the
values of the yield strain, but also change the shape of the \( G' \) curve following the initial linear regime. In the presence of \( \varepsilon-(\gamma\text{-glutamyl})\text{lysine} \) bonds, \( G' \) decreases gradually with strain, up to the fracture point where an abrupt change in \( G' \) occurs due to network breakdown. However, in case of gels without enzyme treatment (i.e., only polymerized via disulphide bonding), a steep change in \( G' \) is noticed.

**CONCLUSIONS**

Enzymatic treatment of whey proteins by transglutaminase provides a facile way to produce gels with controlled rheology at low pH. Samples treated with enzyme exhibited increased elastic modulus and yield strain compared with gels with no enzyme treatment. Enzyme-treated gels were more resistant to decrease of \( G' \) with acidification past the pl. Both gels showed similar mechanical spectra over a wide range of frequency suggesting possible semblance in microstructure. SEM micrographs of both gels corroborates this notion as both gels exhibit similar microstructure.

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