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Rheology of protein gels synthesized through a combined enzymatic and heat treatment method

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Abstract

Whey protein gels prepared under acidic conditions (pH < 4.6) remain largely unutilized because of their weak and brittle nature in contrast to the favorable elastic gels produced at neutral or basic conditions. However, such usage is important, as low pH food products are desirable due to their shelf stability and less stringent sterilization processes. In this study, we use a two-step process involving enzyme followed by heat treatment to produce whey protein gels at low pH (4.0). Dynamic rheological measurements reveal that the gel elastic modulus and yield stress increase substantially when heat treatment is supplemented with enzyme treatment. Both the elastic modulus and yield stress increase with increasing enzyme concentration or treatment time. In contrast, the dynamic yield strain decreases with enzyme concentration but increases with time of enzyme treatment. These results are explained in terms of the enzyme treatment time affecting the diffusion of the enzyme within the gel. This in turn leads to two types of gel microstructure at short and long enzyme treatment times, with the extent of enzyme diffusion modulating the structure at intermediate times.

Keywords: Whey protein; Rheology; Gels; Enzyme; Low pH

1. Introduction

The gelation of polymers into a network structure with specific macroscopic properties has long been an area of research in polymer science and engineering. Of particular interest is the area where specific, biological based modifications can be made, such as through the use of enzymes. Enzymes can provide highly specific modifications in the microstructure of a biopolymer, which can result in significant changes in the macroscopic properties [1].

Enzymes, such as transglutaminase (EC 2.3.2.13), are used to cross-link proteins to form a biopolymer gel. An example of this use is the cross-linking of whey proteins that are used in food products. Transglutaminase catalyzes the formation of cross-links between two proteins by creating an ε-(γ-glutamyl) lysine bond between the γ-carboxyamide group and the ε-amino group of a peptide bound lysyl residue [2]. This allows the formation of new chemical cross-links that would be insensitive to pH. Increases in the number of chemical cross-links would allow control (enhancement) of the fracture stress/strain, vis-à-vis, texture diversity and shelf life, in proteins. Unfortunately, a limitation of this technique is the requirement of prior denaturation of the whey proteins, usually by the addition of chaotropes or denaturants. Therefore, whey protein gels are typically synthesized via heat treatment, which denatures the proteins and promotes thermal reconstruction of the proteins to develop physical and chemical (disulfide) intermolecular cross-links [3,4]. Proteases are another class of enzymes, which can modify the characteristics of whey protein gels [5]. Pretreatment of the whey protein with proteases before heat treatment shows an increase in the cohesiveness and hardness of the gels. One advantage of proteases is the reduced cost and availability, in-contrast to transglutaminase.

Abbreviations: G', elastic modulus; G'', viscous modulus; LVE, linear viscoelastic; P5P, pyridoxal 5-phosphate.

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Four types of intermolecular interactions control whey protein gel formation: hydrophobic interactions (5–10 kJ/mol), hydrogen bonds (10–40 kJ/mol), electrostatic interactions (25–80 kJ/mol) and covalent bonds (200–400 kJ/mol) [2]. Intermolecular hydrophobic interactions arise when previously hidden non-polar amino acid side-chains are exposed as the macromolecular structure uncoils during thermal denaturation. Hydrogen bonding results from the interaction of polar amino acid side-chains. Repulsive electrostatic interactions aid in the production of fine-stranded network structures with good water-holding characteristics. Intermolecular covalent bonding results in permanent cross-links and is most commonly formed by sulfydryl–disulfide interchange [3]. The texture of whey protein gels is controlled by the balance between the weak, reversible 'physical' cross-links (hydrophobic, hydrogen bonding, electrostatic) and the strong, covalent 'chemical' cross-links [3]. Unfortunately, there is no way to tailor the extent of chemical cross-linking during heat treatment [3].

Many fundamental issues related to heat treatment, gelation, and disulfide linkages are known as well as how to develop gels with controlled and enhanced properties [6–8]. However, an overlooked area is the development and utilization of whey protein gels at acidic or low pH (~ 4) because of their weak and brittle nature compared with elastic gels produced at neutral or basic conditions. Presumably, low pH prevents the formation of intermolecular disulfide bonds, which is the only form of chemical cross-links produced during heat treatment. However, another hypothesis is that the electrostatic interactions in whey proteins affect gelation at this pH. Whey proteins have an approximate pI of 4–5 and gels produced by heat treatment at this pH are weaker than those produced at lower pH such as a pH of 1–2 [6]. Although a fundamental understanding of this problem is not complete, the ability to produce a whey protein gel at low pH with desirable texture and rheology would enhance its utility, particularly due to the fact that low pH food products are desirable due to their shelf stability [6] and less stringent sterilization processes [9].

A combination of enzymatic and heat treatments may provide a solution to creating low pH whey protein gels, because it eliminates the weakness found in both methods. A weakness of the enzymatic treatment method is that whey proteins must be denatured to some extent to allow the enzyme access to key amino acids. Acidic pH may partially, if not completely, denature the whey proteins. A weakness of the heat treatment method at low pH is the lack of chemical cross-linking. This may be overcome by enzyme modification.

The aim of this study was to gain physical insight to whey protein gels formed at low pH (~ 4) by using enzyme modification followed by heat treatment. The rationale is as follows. By working at low pH, whey protein should denature thereby facilitating enzyme-catalyzed bond formation. Based on the enzyme selected, modification could be independent of pH. The heat treatment method might serve to deactivate the enzyme as well as allow formation of additional physical cross-links. By understanding the factors affecting enzyme modification and controlling the balance of enzyme and heat treatment, we envision development of low pH gels with tailored textures that mimic neutral and basic pH gels.

2. Materials and methods

2.1. Materials

Samples of whey protein isolate (BiPRO Lot JE 017-8-420) were provided by Davisco Foods International, Inc. (Le Sueur, MN). Microbial 'broad-range' transaminase (T-7684), SealPlate film (Z36,965-9) and pyridoxal 5-phosphate (P-9255) were purchased from Sigma (St. Louis, MO). Costar 12 well Cell Culture plates were used as molds for the gels. Degassed 20% (w/w) whey protein solutions were prepared in water, pH was adjusted with NaOH or HCl (0.1 or 1 M) and NaN_3 added (0.02%) to prevent bacterial growth. All experimental data, except those reported in Fig. 2, were obtained at pH 4.

2.2. Enzyme treatment

The whey protein solutions were placed in a gel mold and incubated at 40 °C for 40 min in a shaking water bath at approximately 50 rpm, unless otherwise specified. The amount of enzyme added was 2.5 U/ml in the waiting time experiment and 5 U/ml for experiments in which enzyme treatment time varied. In all other cases, the enzyme concentration is stated in the figure legends. A stock cofactor solution of 6.3 μM pyridoxal 5-phosphate (P5P) was used to maintain the molar ratio of enzyme to cofactor of 1:5. SealPlate film was used to create a tight, waterproof seal, preventing sample cross-contamination and evaporation. Immediately following enzyme modification, the whey protein samples were placed in an 80 °C water bath for 20 min, then placed at 8 °C for 24 h.

2.3. Dynamic rheological measurements

Dynamic small amplitude oscillatory shear experiments were conducted at 25 °C using a Rheometrics Dynamic Stress Rheometer (DSR II) with either a 25 mm cone and plate (cone angle of 5°) or parallel plate geometry. In this experimental technique, a sinusoidally varying strain $\gamma = \gamma_o \sin(\omega t)$ is applied to a sample,

where γ is the strain, γ_o is the strain-amplitude (i.e. the maximum applied deformation), ω is the frequency of oscillations and t is the time. The response of the sample (a sinusoidal stress, $\tau = \tau_o \sin(\omega t + \delta)$, where τ is the stress, τ_o is the stress-amplitude and δ is the phase angle) is decoupled into an in-phase and an out-of-phase component:

$$\tau = G'\gamma_o \sin(\omega t) + G''\gamma_o \cos(\omega t)$$

The stress in phase with the strain is related through the elastic or storage modulus (G') whereas the stress out of phase with the deformation is related through the viscous or loss modulus (G''). The frequency dependence of G' and G'', together with their magnitude, is a major indicator of microstructure/state (e.g. gel, suspension, solution) of a system. The dynamic frequency spectrum of the modulii were obtained at small strain levels within the linear viscoelastic (LVE) regime to ensure measurement of material properties in at-rest conditions. All reported data were reproducible within 10%. The limits of LVE were determined for all samples by conducting a stress sweep at 1 rad/s.

3. Results and discussion

3.1. Enzyme selection and analysis

Enzyme selection is a key factor in designing a process that can produce a protein gel which has industrial relevance. For an enzyme to be successful in the manufacturing of whey protein gels, it must be produced from a microbial source. The enzyme must also either not require a cofactor or the cofactor must be benign to the gelation process. Thus, a microbial 'broad-range' transaminase was selected that could meet these two conditions and affect the rheology of whey protein gels at pH 4. A two-dimensional gel electrophoresis [10] (performed at Kendrik Laboratories, WI) revealed transaminase had a pI of 6.0 and a molecular weight of 40 kDa. Conventional sodium dodecyl sulfate polyacrylamide gel electrophoresis [11] indicated the enzyme had a molecular weight of 36.8 kDa. The average of these values was used in our calculations. Since P5P concentration did not affect G' of whey protein gels (Fig. 1), it appears that this cofactor does not have any effect on the whey protein gelation.

Transaminases are a class of enzymes which have not been previously used to modify whey protein gels; however, the macroscopic effect was clearly evident based on preliminary rheology experiments with this system (data not shown). In the literature [12,13], broad-range transaminases have been shown to accommodate poor substrates that have undergone transaldimination, whereby an external aldimine is created between the P5P and the substrate [14]. If this reaction occurred with the whey protein and P5P, or if the transaminase reacted directly on an amino acid such as a glutamine, it is clear that the electrostatics of the biopolymer would change. Further biochemical analysis is required to elucidate the microstructural changes of the whey protein gels upon treatment with the broad range transaminase, and will be addressed in a future study.

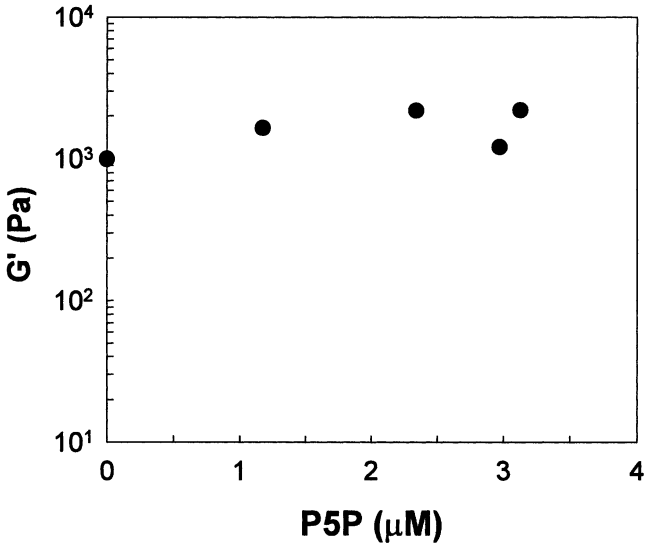

Fig. 1. G' vs. P5P concentration for whey protein gels produced using heat treatment only.

3.2. Effect of pH on rheological properties

The mechanism of whey protein gelation at low pH has not been fully understood to date [15–18]. Heat treatment of whey proteins allows the formation of intermolecular disulfide bonds. However, sulfhydryl–disulfide exchange is sensitive to pH [8]. Acidic pH reduces G' approximately two orders of magnitude below values obtained at neutral pH (Fig. 2). The pH dependence has also been studied by using fracture stress and strain analysis of whey protein gels and display the same trends as that observed in Fig. 2 [19]. The weak, brittle gels obtained at low pH ($\sim$4) might result from an absence of disulfide bonds [6]. Support for this conclusion comes from the fact that the addition of disulfide reducing agents (dithiothreitol or β-mercaptoethanol) at neutral pH transforms gels from elastic to brittle [20,21] and similar results are observed when the pH is adjusted from neutral to low pH ($\sim$4) [6,22]. As the pH of the solution reaches the isoelectric point of the proteins (whey protein is 55–62% β-lactoglobulin which has a pI = 5.2), the structure of the gels collapse producing a weak coagulate [23–25]. Another factor contributing to low G' at low pH may be the propensity of whey protein solutions to form less covalent cross-links with decreasing pH [8].

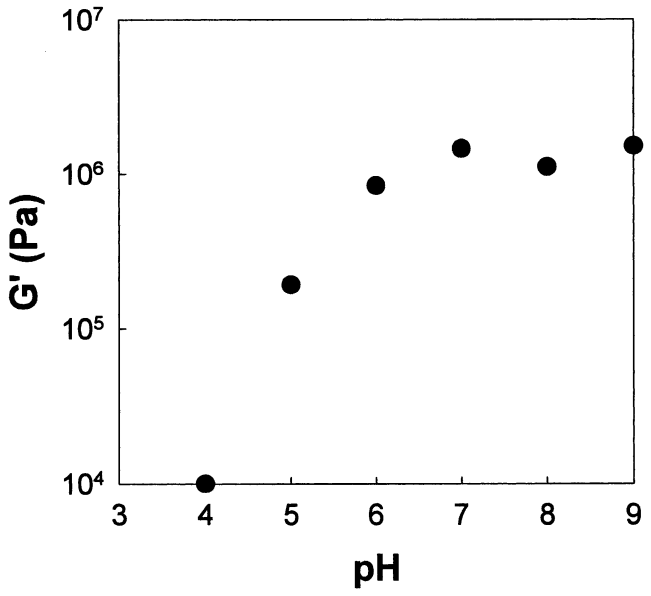

Fig. 2. G' of whey protein gels produced after 20 min at 80 °C at varied pH.

3.3. Enzyme treatment

When whey protein solutions were treated with transaminase followed by heat, both G' and G'' increased more than one order of magnitude over values obtained for heat treatment alone (Fig. 3). This suggests that the enzyme pre-treatment yielded additional cross-links.

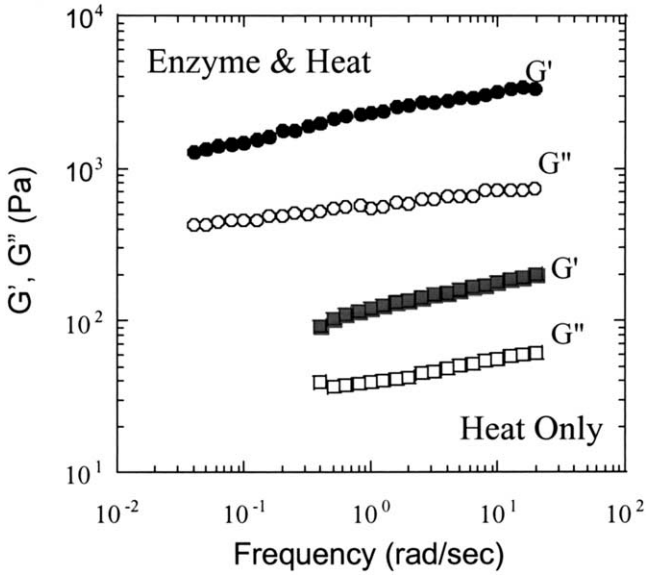

Fig. 3. Dynamic frequency spectra of G' and G'' for heat-treated whey protein gels (squares) compared with that of combined enzyme and heat-treated gels (circles). A two step heat treatment method was used: 40 min at 40 °C followed by 20 min at 80 °C. The combined treatment method was the same except 5 U/ml of transaminase was added at the beginning of the first heat treatment step.

While G' is an effective parameter to measure the overall strength of a gel, other rheological properties are more closely related to the texture of the gel. Two properties, which are traditionally measured during the fracture of a material, are the fracture stress and fracture strain. These properties have been qualitatively linked to the texture and 'mouthfeel' of the gel [26]. In addition to using large fracture stress and strain, dynamic experiments can also be performed that will detect the limit of linearity by the dynamic yield stress or strain (Fig. 4). Values for the dynamic yield stress and strain follow the same trends as the large fracture (steady) yield stress and strain values [27,28]. Thus, for qualitative purposes such as those relating to texture, it is appropriate to use either the fracture or dynamic stress and strain at the yield point. An advantage to using dynamic yield stress and strain is the ability to apply previously developed fractal models to our results [7]. The fractal analysis allows the elastic properties of the whey protein gel to be related to its network structure.

When G' and G'' are compared for heat and enzyme-heat treated samples as a function of increasing stress amplitude, G' initially dominates until a critical stress akin to a fracture stress is reached (Fig. 4). A structural breakdown leading to catastrophic reduction in moduli occur beyond this critical point. However, the critical stress increases by more than an order of magnitude when a combined heat and enzyme treatment is used. An interesting point that was noted during these preliminary experiments was that the time waited

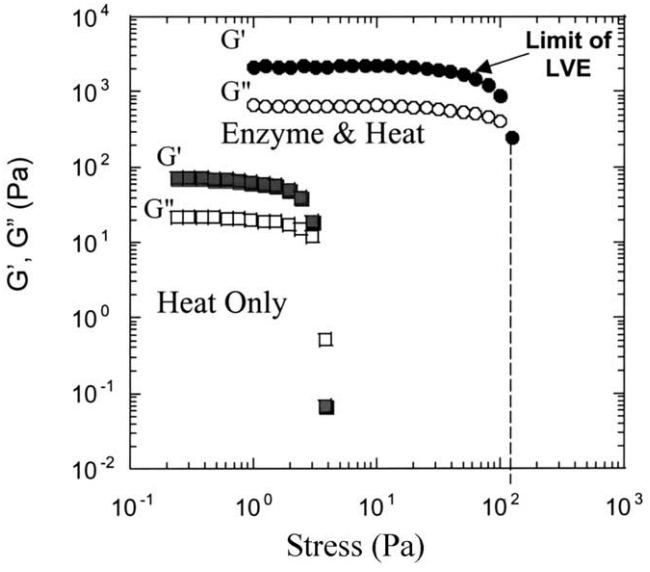

Fig. 4. G' and G'' as a function of increasing stress amplitude shown for a heat treated whey protein gel and a combined enzyme and heat treated whey protein gel. A two-step heat treatment procedure was used: 40 min for 40 °C followed by 20 min at 80 °C. The combined enzyme and heat treatment procedure was the same except 5 U/ml of transaminase was added at the beginning of the first heat treatment step.

between whey protein dissolution at pH 4 and enzyme treatment affected the final rheological properties (described below).

3.4. Effect of waiting time

Denaturation of whey protein with time at pH 4 occurs due to loss of electrostatic interactions, because this pH is very similar to the pI of the prominent whey protein component, β-lactoglobulin. α-Lactoalbumin, another component of whey, changes to a molten globule state between pH 2 and 4 with a hydrodynamic volume around 15% larger than the native state [15,29]. We investigated the denaturation effect by dissolving whey proteins at pH 4 and waiting various periods of time (at room temperature) before enzyme and heat treatment. There was an order of magnitude increase in G' when the solution was allowed to sit for 1 day (Fig. 5). Beyond this time, there was not any additional increase in the G', suggesting the fullest extent of pH-dependent denaturation was achieved within 24 h or less.

3.5. Effect of enzyme concentration on the G′

While a combination of enzyme and heat treatment at low pH dramatically increased G', the results at pH 4 do not match the values found at pH 7. To achieve this goal, we modified the processing conditions. In classical chemical cross-linked polymer systems, the number of cross-links in a gel is proportional to the G' [30,31]. Therefore, we surmised that additional quantities of the enzyme would create more cross-links and increase G'.

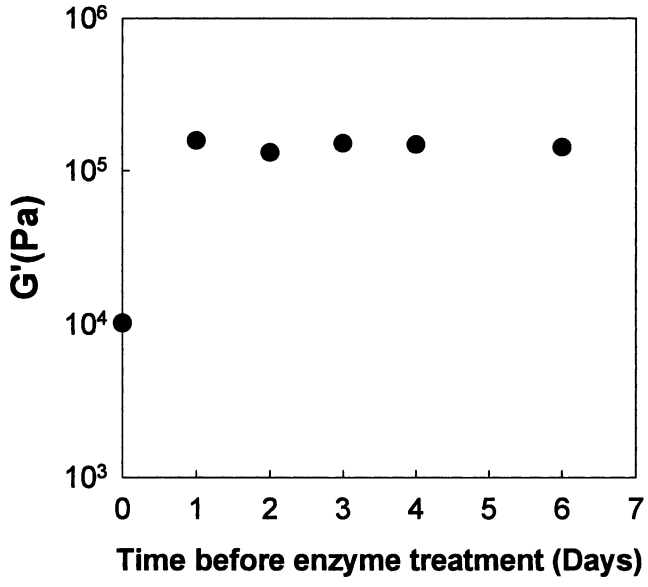

Fig. 5. G' of whey protein gels obtained after various equilibration times at pH 4. The whey protein gels were produced by enzyme cross-linking with 2.5 U/ml of transaminase followed by heat treatment.

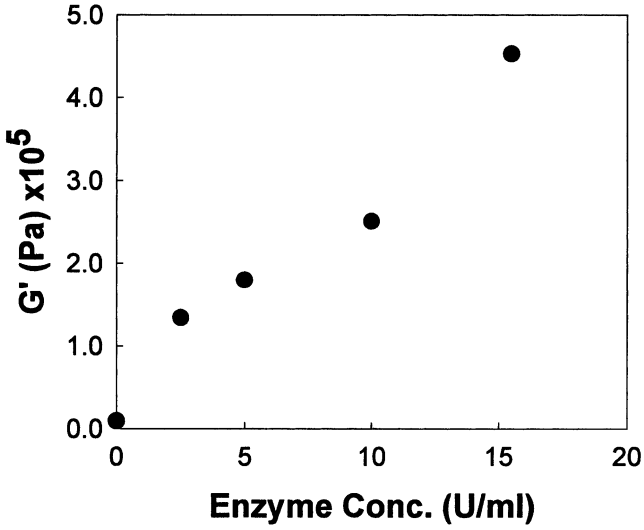

Fig. 6. The effect of transaminase concentration on G' of whey protein gels produced from a combined enzyme and heat treatment method. Enzyme treatment was for 40 min for 40 °C followed by 20 min at 80 °C.

In fact, additional enzyme generated a proportional increase in G' values (Fig. 6). This result allows us to a priori predict the final rheological properties of the whey protein gels and tailor their gel structure to match the food product it will be used in.

The effect of enzyme concentration on the dynamic yield stress were similar to G'; the dynamic yield stress increased linearly with enzyme concentration (Fig. 7). However, the dynamic yield strain results were dramatically different; increased enzyme concentration decreased the yield strain (Fig. 8). The yield strain of whey protein gels decreases as the network phase volume is increased [7,32]. Simply put, if there is an

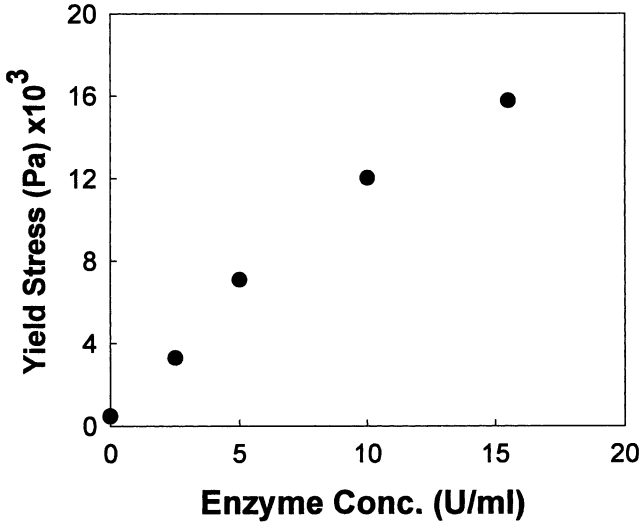

Fig. 7. Dynamic yield stress as a function of transaminase concentration for whey protein gels prepared through combined enzymatic and heat treatment.

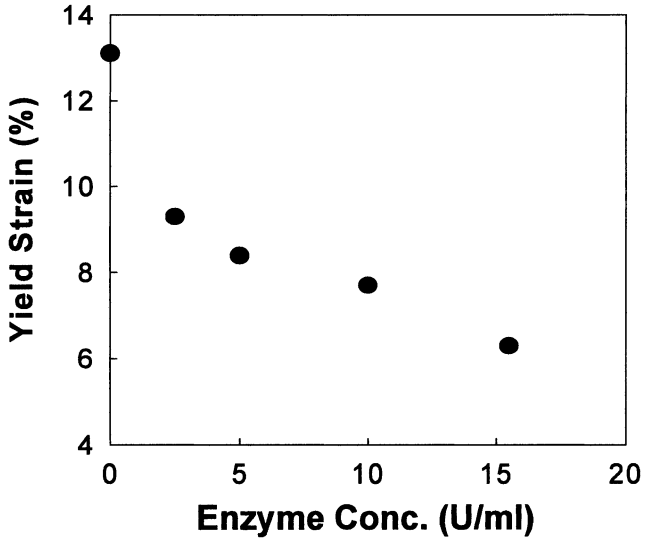

Fig. 8. The effect of transaminase concentration on the dynamic yield strain of whey protein gels prepared through combined enzymatic and heat treatment.

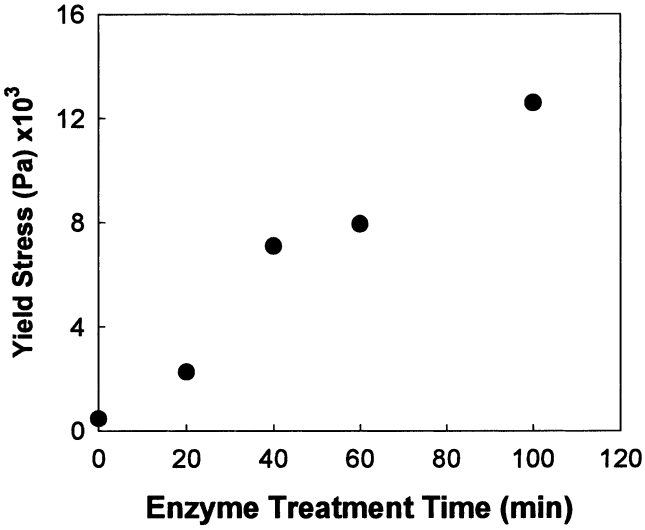

Fig. 9. Dynamic yield stress as a function of time of transaminase treatment for whey protein gels prepared by enzyme and heat treatment. Enzyme concentration was 5 U/ml.

abundance of weak bonds, not much strain needs to be applied before yielding is observed. We can interpret these results based on fractal models [33–36]. The model of Shih et al. [36] assumes a gel network is a collection of closely packed fractal flocs with two possible modes of behavior depending on the relative strengths of the inter- or intra-floc bonds. Based on this model, whey protein gel is a strong-link gel [7] in which the network has higher elasticity in the links between neighboring flocs (inter-floc) than those within the floc (intra-floc) [36]. A decrease in the yield strain concurrent with an increase in network phase volume is a typical response of a strong-link gel versus a weak-link gel which shows an increase in the yield strain. The same logic suggests that the network structure of our gel continues to be of the strong-link type. The fractal model of Bremer et al. [33–35], on the other hand, is based on the deformation mechanism of the network strands. In this regard, the whey protein gel is classified as a Type 2 gel in which the network bends in response to an applied stress rather than stretches (Type 1 gel) before fracture, and exhibits a decreasing yield strain with increasing phase volume [33–35].

3.6. Effect of enzyme treatment time

In an effort to increase both the dynamic yield stress and strain, we examined the effects of other processing conditions that might produce the desired result. For example, the dynamic yield stress increased linearly with longer enzyme treatment times (Fig. 9). This is similar to the response found by increasing the enzyme concentration at short treatment times (Fig. 7).

Enzyme treatment results in a somewhat constant yield strain to treatment times of up to 40 min whereupon the yield strain increases quite rapidly (Fig. 10). This trend in the yield strain is analogous to the behavior of egg white gels in which the fractal analysis was applied to further characterize the gel network structure [7]. Using Bremer's model [7], the egg white was characterized as a Type 1 gel where the protein strands are stretched under applied stress rather than bent (Type 2 gels) and the yield strain increases with protein concentration. The increase in yield strain with enzyme treatment time for whey protein gels, therefore, suggests a change from a Type 2 to a Type 1 gel. From

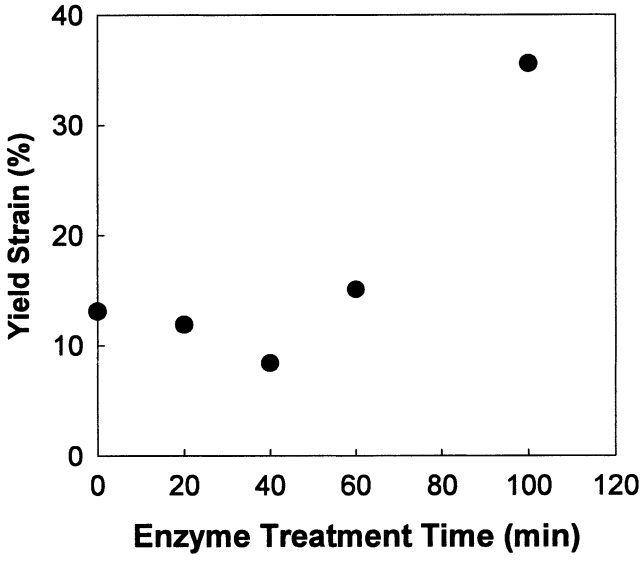

Fig. 10. The effect of tansaminase treatment time on the dynamic yield strain of whey protein gels prepared by enzyme and heat treatment. Enzyme concentration was 5 U/ml.

the standpoint of the model of Shih et al. [36], the yield strain decreases with increasing network phase volume in the strong-link gel but increases for a weak-link gel [36,37]. Whey protein gels were characterized as strong-link gels but a transition to weak-link occurs as the enzyme treatment time is increased (Fig. 10). The weak-link gel is characterized by stronger intra-floc bonds versus inter-floc bonds. The ability to adjust key processing parameters (such as the enzyme treatment time) should enable us to tailor the final rheological and textural properties of the whey protein gels.

We believe that the diffusion of the enzyme throughout the whey protein solution, before thermal gelation, plays a key role in determining the final structure of the gel networks. When short enzyme treatment times are used, the enzyme may not have sufficient time to diffuse throughout the gel leading predominantly to inter-floc bonds with few intra-floc bonds; this is reminiscent of a strong-link gel. Even if the enzyme concentration is increased, the short treatment time limits diffusion so the increase in elasticity is found in the inter-floc bonds and limited intra-floc bonds result in the low yield strain (Fig. 8). When time is increased such that the enzyme has more time to diffuse, both inter- and intra-floc bonds can be created, providing more cross-links throughout the entire gel network (behavior akin to a weak-link gel). This results in a more overall elastic character as opposed to distinct regions of high elasticity. Therefore, the yield strain (Fig. 10) is increased as well as the other rheological properties such as the yield stress and the elastic modulus.

While we have characterized varied processing conditions on the final rheological properties of whey protein gels, the mechanism of enzyme modification has not been elucidated. The rheological results suggest that the enzyme is able to create bonds at a rate proportional to extent of enzyme accessibility, which suggests chemical cross-links are created. However, these issues are beyond the scope of this work and will form part of subsequent research that deals with the mechanism of the enzyme modification. Nevertheless, the results obtained in this study provides a major step in understanding the effect of processing parameters on the rheological properties of whey protein gels and expanding the range of possible textures of whey protein gels formed at pH 4.

4. Conclusions

Enzyme modification of whey proteins is an effective tool to tailor the final rheological properties of a whey protein gel at low pH. A methodology was developed to combine enzyme and heat treatment to produce whey protein gels at a low pH. Several process parameters including enzyme concentration, enzyme treatment time and waiting time used in this methodology were modulated to determine their effect on the final gel properties. After whey protein dissolution at pH 4, the elastic modulus showed an order of magnitude increase if a certain time lag (24 h) was used before enzyme treatment. This waiting period possibly enabled denaturing of the protein thereby providing easier access for the enzyme. Both the elastic modulus and the yield stress increased linearly with enzyme concentration in contrast to the yield strain, which showed a decreasing trend. Increasing the enzyme treatment time resulted in a monotonic increase in both the elastic modulus and yield stress. The yield strain remained approximately constant with increasing enzyme treatment time until up to 40 min of treatment, whereupon the yield strain began to increase considerably. These results are explained in terms of the diffusion of the enzyme playing a major role in the gel structure with the gel structure changing from a ‘strong-link’ gel at short enzyme treatment times to a ‘weak-link’ gel after long enzyme treatment times.

Acknowledgements

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