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ORIGINAL PAPER

Gelling Properties of Tyrosinase-Treated Dairy Proteins

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Abstract Protein-based viscous gels can augment or replace carbohydrate-based ones for specific nutritional formulations such as in reduced calorie or low-fat food applications. In this study, slurries of whey protein isolates and calcium caseinate mixed with alginic acid (20% T.S.) were subjected to high-shear homogenization (microparticulation) at 27,000 rpm for 2, 3, 4, and 6 min. The resulting slurries were incubated with mushroom tyrosinase (E.C. 1.14.18.1) at levels of 3, 6, and 9 mg/100 g for 15, 30, and 60 min to facilitate gel formation of the alginic acid with the homogenized dairy proteins. The results indicate that the time of high-shear homogenization had significant ($P<$ 0.05) effect on the viscosities of the gels. Highest gel viscosity was obtained with 6 mg tyrosinase at 60 min, but increases in gel viscosity depended on time of shear, with 2 and 4 min shear resulting in higher viscosity (484 and 6,143 cP) and stronger complex viscosity (49 and 38 Pa.s at 1 rad/s), respectively, compared to the control (69 cP) and (12 Pa.s at 1 rad/s). Gels were stable in refrigerated storage up to 240 h, strengthened with time of refrigeration storage, and became significantly more viscoelastic. Optimal viscous properties were obtained at 4 min microparticulation, 60 min incubation, and 6 mg tyrosinase treatment.

Keywords Enzyme · Tyrosinase · Gels · Microparticulation · Whey proteins

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Introduction

Microparticulation is a process that combines thermal and mechanical shear processes using high homogenization to change the natural small spherical structural conformation of proteins to random coils (Singer and Dunn 1990). This process was demonstrated by heating egg and whey protein solutions in between 90°C and 120°C, at pH between 3.7 and 4.2, and shear rates ranging from 450,000 to 600,000 reciprocal minutes, for about 5 min. Microparticulation reduces sizes of the proteins to less than 3-μm spheres (Singer et al. 1988). The nearly spherical structure is the basis for protein-based fat substitutes, such as Simplesse™, which has creamy, smooth emulsion-like feel and fat-like organoleptic effects, but contributes no viscoelastic properties to formulations.

Gels created by microparticulation and cooking of mixtures of proteins are mostly heterogeneous mixtures with interpenetrating networks; their viscoelastic properties come only from changes in their microstructure (Zeigler 1991). Microparticulation of whey proteins creates low-density matrices with increased surface area, which produce soft gels (Doi 1993). Due to their poor gelling ability, the protein-based fat replacers sometimes are combined with food gums, such as guar gum, to enhance their binding properties (McMahon et al. 1996). In our previous work, we showed that calcium caseinate and whey protein concentrates containing 21% sucrose by weight sheared at 27,000 reciprocal minutes with sizes ranging from 30 to 300 μm formed very soft gels (Onwulata et al. 2002).

Stable gels are sometimes created by microparticulation and evaporative cooking processes that increase the interaction of protein and other adjuncts such as polysaccharides (Bernal et al. 1987). Transglutaminase (EC 2.3.2.13) is an enzyme commonly used to crosslink food proteins to enhance the functional benefits such as

increased gel stiffness, gelation capability, thermal stability, and water-holding capacity of meat proteins. Transglutaminase is now widely used in seafood, surimi products, meat products, noodles or pasta, and dairy products to improve firmness, elasticity, and viscosity (Kuraishi et al. 2001). However, whey proteins as isolates or in purified form [beta-lactoglobulins (β-LG)] cannot be crosslinked by transglutaminase because of their globular nature, except when unfolded or denatured. Crosslinking is also further limited by non-covalent aggregates formed between Ca^{2+} and β-LG induced by electrostatic interactions (Faergemand et al. 1998). Tolkach and Kulozik (2005) showed that whey proteins are less sensitive to transglutaminase and are not crosslinked; only the casein fractions aggregated slowly.

Milk protein and alginic acid can be processed through microparticulation and heated to form soft gels (McClements et al. 1993). In Onwulata et al. (2002), it was shown that microparticulation of whey protein with sucrose reduced particle sizes from 800 to 180 μm, which resulted in softer gels as time of shearing increased. Research on milk protein and alginic acid microparticulation to create composite gels is very limited, but the opportunity exists for making co-products or conjugated gels linked with other polymers using other enzymes, for example, tyrosinase (EC 1.14.18.1). Tyrosinase catalyzes oxidative covalent crosslinking between two phenolic rings, one from tyrosine and an amino group (Seo et al. 2003). For example, tyrosinase was used to catalyze crosslinking of chicken myofibril proteins forming a thermally stable gel (Lantto et al. 2006).

Lantto et al. (2005) showed that, the mushroom enzyme, tyrosinase increased gelation of fibrous wool proteins giving products with improved physical properties. Particularly, they enhanced gel strengths (Bloom), increased melting points, viscosities, and temperature of gelation. Tyrosinase enzymes are able to crosslink peptides of 3–10 kDa derived from enzymatically hydrolyzed wool fibers. Since cross-links are favored in denatured globular proteins, we investigated the use of tyrosinase enzyme from mushroom to increase the gel strength of whey protein isolate (WPI) and calcium caseinate denatured by high-shear homogenization (HSH) in the presence of alginic acid and the rheology of composite gels created through thermal evaporation.

Materials and Methods

Gel Preparation

Whey protein isolate (Provon 190) and calcium caseinate (Alanate 391) were purchased from New Zealand Milk Products (Santa Rosa, CA, USA); alginic acid and mushroom tyrosinase (TNase) were purchased from Sigma Chemical (St. Louis, MO, USA). Composite slurry was prepared by mixing 40 g of WPI and 40 g of calcium caseinate with 320 g of finely crushed ice and blending for 24 s, using a Waring Lab Micronizer (Waring Products Division, New Hartford, CT, USA). Then, 2 g alginic acid was added.

High-Shear Homogenization

The composite slurry (CS), milk proteins plus alginic acid, was then microparticulated by high-shear homogenization (HSH) using a PCU-2 homogenizer (Brinkman Instruments, Westbury, NY, USA) for 2, 3, 4, or 6 min. Before evaporation, the HSH slurry was incubated with 3, 6, or 9 mg tyrosinase for 15, 30, or 60 min at 30°C. After incubation, the HSH slurries were then evaporated to form gels by heating at 45°C for 40 min under partial vacuum (5.2 MPa) in a Rotary Evaporator Model RE-52 (Yamato Scientific, Tokyo, Japan). The gels after evaporation were 13% to 15% solids. Three evaporations yielded 300 ml of shear-treated gel, which was divided into three aliquots. The first was used for determining viscoelastic properties at ambient temperature within 4 h of completion of evaporation for day-0 values. The second was used for storage studies. The gels were stored at 4°C for up to 300 h, and gel properties were evaluated at several stages. A portion of the day-0 gels was frozen at −22°C for 7 days and thawed for 4 h at 22±0.2°C to evaluate freeze thaw stability of the gels. The third portion of the gels was freeze-dried (~90% solids) and was reconstituted at a ratio of 0.15 g freeze-dried gel per milliliter of water at room temperature for 2 h before determining rehydrated gel strength.

Experimental Design

A completely randomized design with a factorial treatment was used to evaluate the time effects of HSH and tyrosinase treatment on formulated gels. Two replications of the 4×2 factorial design, four HSH conditions 0, 2, 4, and 6 min, respectively, and two gel preparations with 6 mg/100 g and without (0/100 g) tyrosinase constituted the full experiment. Analysis of variance was used to identify differences in physical properties at various processing conditions. Bonferonni's least significant difference was used to test for mean separation. The SAS statistical software package was used (SAS Institute, Cary, NC, USA) in all cases. Significance levels were defined at $p<0.05$.

Gels made from evaporated slurry were analyzed after 2 h at 24°C for day 0, then refrigerated at 4°C, or frozen (−22°C) for analyses. Total solids values were determined from moisture analysis using AOAC Method 991.42 (AOAC 1998).

Table 1 Effect of time of incubation on viscosity of composite gels of dairy proteins and alginic acid

Enzyme (mg)	Time of incubation (min)		
	15	30	60
	cP		
0	155 b	190 c	224 c
3	311 a	279 b	1,145 b
6	308 a	432 a	2,000 a
9	124 c	–[a]	–[a]

Pooled standard error for gels: (11.9). Means of three replicate per sample. Samples with different letters within a column are different from one another by Bonferroni's multiple range test. Moisture of gels ranged from 85% to 87%.
cP Centipoises
[a] Gels were too clumpy to measure.

Viscosity Determination

Viscosity was determined at 22±0.2°C using a Brookfield Digital Rheometer model DV–III (Stoughton, MA, USA). The viscosities of the evaporated composite gels were determined using spindle number 3, effective length of 4.946 cm, and very soft gels were determined using the ULA spindle, effective length of 9.239 cm. For analysis, 50 ml of each sample was used.

A strain test was made to evaluate product behavior at constant frequency, identifying the range of linear visco-elasticity from 0.1% to 2% strain. A frequency test was made to measure viscous stability and dynamic viscous response to shear and temperature at oscillation frequency (ω) ranging from 0.01 to 100 rad/s with fixed amplitude of the protein–alginic acid composites. The tests were performed at 18.50±0.05°C with a Rheometrics RDA-700 Dynamic Analyzer (Rheometrics, Piscataway, NJ, USA) equipped with a 200 g/cm torque transducer using 2.0-cm radius parallel plates with a separation distance of 0.2 cm. Change in viscoelastic properties as a function of time and temperature was determined by dynamic shear measurement. The measurement gave the shear storage modulus (G') and loss modulus (G''). Complex viscosity (η^*) was determined from the frequency sweep in a range from 1 to 100 rad/s. The gel samples were analyzed twice for each of the three replicates.

Results

To increase the strength of dairy composite gels, the composite slurries (CS) were incubated with varying amounts of TNase/100 g CS for varying times post-HSH (Table 1). Viscosity increased with time of incubation and concentration of TNase. Flowable, non-lumpy gels were obtained with 6 mg/100 g TNase with optimal time of incubation of 30 min, yielding the highest measured value. Enzyme concentration effects on gel viscosity were very significant ($p<0.05$) for both time of incubation and concentration. At low frequency (~1 rad/s), the non-enzyme-treated gel (Fig. 1a) exhibited lower range of complex viscosity below 10^2 Pa. There were significant increases ($p<0.05$) in viscosity values for gels incubated with 4 mg tyrosinase (Fig. 1b). The range of values for elastic, loss moduli, and (η^*) was between 10^2 and 10^3 (Pa and Pa.s) for TNase treatment (Fig. 1b). The results show that 30 or 60 min incubation time with 6 mg TNase were better than the rest. Incubating the microparticulated CS for various times ranging from 15 to 60 min generally resulted in increased gel strength with time. Though optimum increase was seen with 6 mg TNase at 60 min, effective composite stabilization was achieved at the reasonable incubation time of 30 min. Anticipating the commercial use of TNase in creating gels, a process with reduced time of incubation, 30 instead of 60 min, may favor its adoption. Process-induced effects on enzyme activity have been reported: for *Kluyveromyces lactis*, increased lysozyme activity at less than optimal pH conditions (Huang and

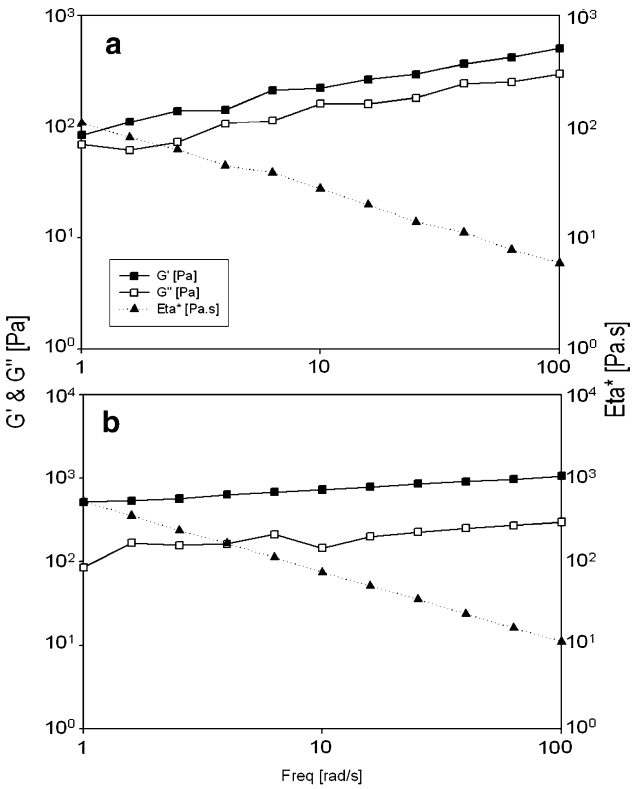

Fig. 1 Plot of the viscoelastic properties of non-shear (**a**), gel conditions: HSH 0 min, 6 mg TNase, 30 min incubation, and HSH gels (**b**), gel conditions: HSH 6 min, 6 mg TNase, 30 min incubation. *Filled square* Elastic modulus (G'); *open square* loss modulus (G''), and *filled triangle*, Eta* (complex viscosity)

Demerei 2008), and for *Lactobacillus bulgaricus*, improved cryotolerance with centrifugation (Streit et al. 2008).

Viscosity increased also with time of shear, increasing at 2 and 4 min, respectively, and decreasing at 6 min (Table 2). HSH gels at 6 min were very weak to measure. Typical viscoelastic behavior in the 10^1 to 10^2 Pa.s ranges at ~1 rad/s are shown in Fig. 2a for no shear gel and in Fig. 2b for 6 min HSH gel. The elastic moduli (G') and complex viscosity (η^*) were not changed, but the loss moduli showed a transition from the shear effect (Fig. 2b). Both elastic (G') and viscous (G'') values, independent of frequency, show solid-like tendencies when G' is greater than G'' and liquid-like behavior when G'' is greater than G'. A transition from one dominant behavior G' or G'' to the other indicates change in network structure. Changes in viscosity may be due to intra- and intermolecular motions related to size of protein aggregates as the proteins coalesce and separate simultaneously. This is the result of complex molecular forces and residual surface-induced changes in the hydrogen-bonding structure (Zhu and Granick 2001). These results are typical, as we have previously reported on the time of shear-dependent effect of microparticulated milk proteins and sucrose (Onwulata et al. 2002).

Stability of the composite gels in refrigerated storage up to 240 h indicated strengthening of all gels with length of storage at 4°C. Tyrosinase-treated gels became significantly more viscous with time of storage (Table 3). The evaporation conditions in the gel-making step, 45°C under partial vacuum (5.2 MPa) for 40 min, may not have been sufficient to stop the activity of tyrosinase. Furthermore, protein self-aggregation with samples without tyrosinase caused increases in viscosity (Table 3; specimens with HSH time labeled with superscripted a). Strength of the gels increased in magnitude with storage (Fig. 3b compared to a), suggesting stronger networks formed. Time of shear-dependent effect of HSH was observed only initially from

Table 2 Effect of HSH and incubation with 6-mg tyrosinase on viscosity of whey proteins and alginic acid composite gels

Microparticulation (min)	Time incubation (min)	
	0	30
	cP	
0	66 c	190 d
2	193 b	484 b
4	224 a	6,143 a
6	–[a]	279 c

Pooled standard error for gels: (15.2). Means of three replicate per sample. Samples with different letters within a column are different from one another by Bonferroni's multiple range test. Moisture of gels ranged from 85% to 87%.
cP Centipoises
[a] Missing data (Weak gels)

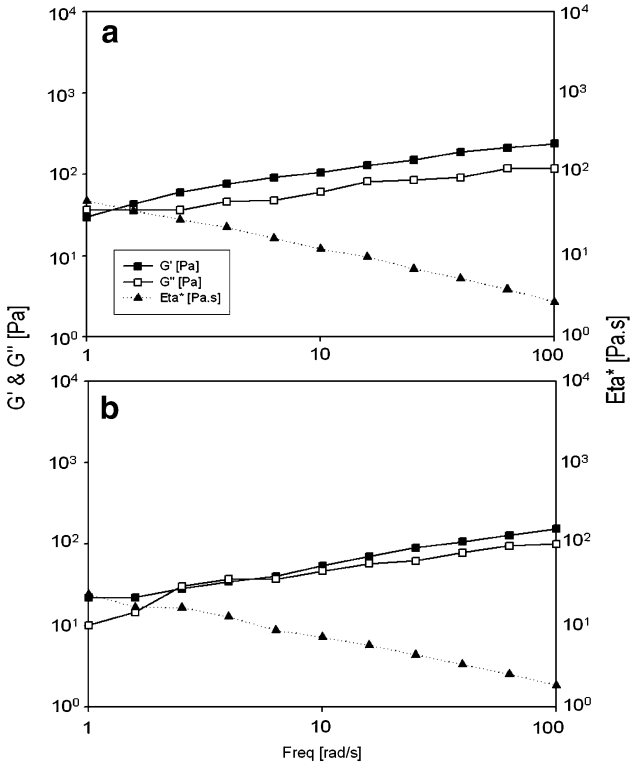

Fig. 2 Plot of the viscoelastic properties gels showing time of shear effects, 6 min (**a**), gel conditions: HSH 6 min, 6 mg TNase, 30 min incubation (**b**), 0 min, gel conditions: HSH 0 min, 6 mg TNase, 30 min incubation. *Filled square* Elastic modulus (G'); *open square* loss modulus (G''), and *filled triangle* Eta* (complex viscosity)

days 1 up to 4; after that, from days 7 to 10, the previously weaker gels became significantly ($p<0.05$) stronger and more viscous (data not shown).

Freeze-dried and reconstituted gels (Fig. 4b) showed storage and loss moduli above that of its control (Fig. 4a). In general, viscoelastic behavior of the gels followed the

Table 3 Effect of HSH with tyrosinase and storage time on viscosity of dairy proteins and alginic acid composite gels

Microparticulation (min)	h	96 h	168 h	240 h
	cP			
0	66 g	254 f	240 g	5 g
0[a]	190 e	3,825 c	12,897 c	28,685 d
2	194 e	199 g	182 h	321 e
2[a]	484 b	1,147 d	1,854 d	89,295 b
4	224 d	315 e	285 f	256 f
4[a]	6,143 a	29,934 a	14,871 b	39,142 c
6	130 f	212 g	952 e	265 f
6[a]	279 c	5,699 b	200,139 a	517,090 a

Pooled standard Error for gels: (11.2). Means of three replicate per sample. Samples with different letters within a column are different from one another by Bonferroni's multiple range test. Moisture of gels ranged from 85% to 87%.
[a] Gels treated with 6 mg tyrosinase, *cP* centipoises

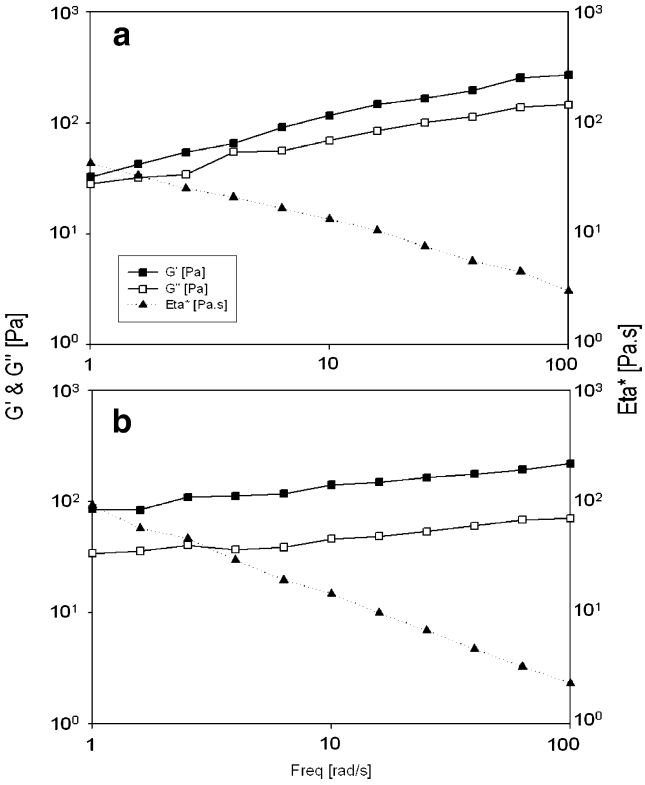

Fig. 3 Plot showing effect of tyrosinase treatment on viscoelastic properties of gels. Top plot (**a**) is non-treated, gel conditions: HSH 0 min, 0 mg TNase, 30 min incubation, and bottom plot (**b**) is treated with 6 mg tyrosinase, gel conditions: HSH 0 min, 6 mg TNase, 30 min incubation. *Filled square* Elastic modulus (G'); *open square* loss modulus (G''), and *filled triangle* Eta* (complex viscosity)

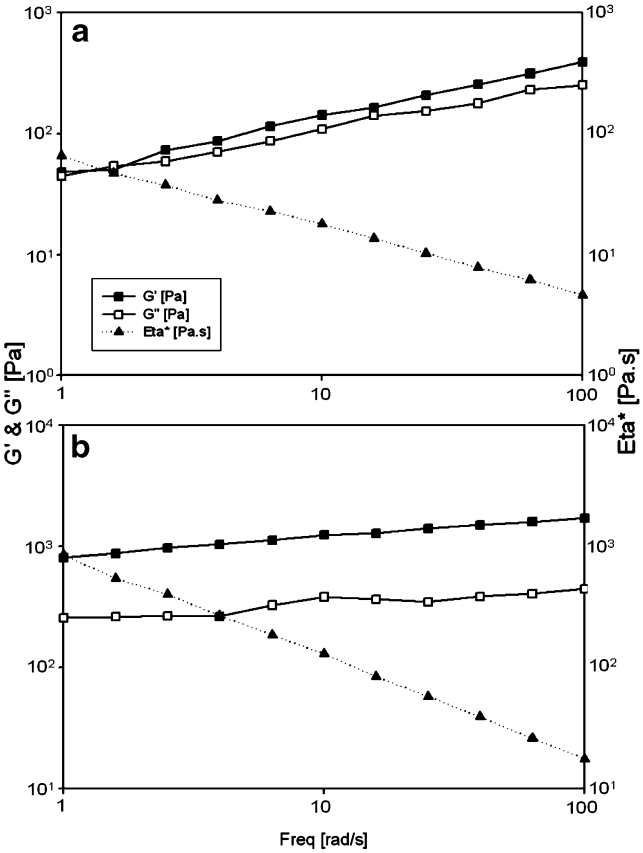

Fig. 4 Plot showing time of storage effect on the viscoelastic properties of gels, day 0 (**a**), gel conditions: HSH 3 min, 6 mg TNase, 30 min incubation and day 4 (**b**), gel conditions: HSH 3 min, 6 mg TNase, 30 min incubation. *Filled square* Elastic modulus (G'); *open square* loss modulus (G''), and *filled triangle* Eta* (complex viscosity)

same pattern of shear dependency and increased elasticity with TNase treatment (Table 4). TNase-treated gels had significantly ($P<0.05$) higher viscosity than the non-treated control gels. Maximum energy storage was at 2 min HSH with TNase incubation. Change in storage modulus was not significant after 2 min, not even with TNase treatment. The viscoelastic property of gels microparticulated at 6 min, showed by the ratio of G''/G', was more solid-like, but this was reversed with TNase treatment.

Discussion

From our previous study with whey protein concentrate, sucrose, and sodium caseinate composites, we showed that microparticulated composite gels exhibited significant reduction in storage modulus and complex viscosity that depended on time of shear (Onwulata et al. 2002). Others have reported also that microparticulated slurries containing superfine structures do not form interpenetrating networks (Taylor and Fryer 1994). It was necessary to investigate the efficacy of a crosslinking enzyme in creating or enhancing gel properties of microparticulated protein–starch complexes. From this work, our finding is that, in general, treating dairy protein composites with tyrosinase in the presence of small amounts of alginic acid increased gel strength. The process of microparticulation provided the

Table 4 Effect of HSH and incubation with tyrosinase on rheological properties of dairy proteins and alginic acid gels

Microparticulation (min)	G' (Pa)	G'' (Pa)	η^* (Pa.s)
0	11.7 f	9.3 e	0.3 d
0[a]	76.3 b	65.6 b	2.0 b
2	48.9 d	34.9 d	1.9 b
2[a]	200.0 a	104.7 a	4.5 a
4	38.3 e	31.9 d	1.0 c
4[a]	69.5 c	61.1 b	1.9 b
6	8.1 f	12.5 e	0.5 c
6[a]	71.3 b	56.6 c	1.8 b

Pooled standard error for gels G' (3.11); G'' (2.69); η^* (0.54). Means of three replicate per sample. Samples with different letters within a column are different from one another by Bonferroni's multiple range test. Moisture of gels ranged from 85% to 87%.

[a] Gels were treated with 6 mg tyrosinase

driving force needed to unfold native protein structures, which in turn initiated and promoted aggregation and protein association through crosslinking of exposed glutamine residues, promoting gel formation (Totosaus et al. 2002). However, weak gels are formed from heavily sheared whey proteins and gelatin (Walkenstrom et al. 1998).

Aggregation behavior had a major effect on the strength of the whey protein gel network. The presence of sugars or polysaccharides with milk proteins intensified aggregation of denatured molecules and promoted gelation (Walkenstrom et al. 1998; Steventon et al. 1994). The use of xanthan gum enhanced covalent crosslinking in a pressure induced gel, creating a fine-meshed structure (Zasypkins et al. 1996). In addition, Walkestrom and Hermansson (1997) showed that mixed gels containing whey proteins and gelatin could be produced by high temperature or shear. Furthermore, gels created from mixtures of proteins and carbohydrate was less sensitive to shear retaining strong loss modulus (Walkenstrom et al. 1998). This may explain the increased gel strength associated with our microparticulated gels containing both dairy proteins and alginic acid.

Polymerization of dairy proteins by enzymatic oxidation is already being used to increase the range of functionality in different products. For example, purification of β-lactoglobulin and α-lactalbumin from a whey protein concentrate that contained casinomacropeptide was accomplished by enzymatic fractionation using transglutaminase, leaving in native whey proteins that were less sensitive to transglutaminase in the serum (Tolkach and Kulozik 2005). Caseins are easily crosslinked with transglutaminase, but not the whey fractions, except when unfolded (Faergemand et al. 1998). Faergemand et al. (1998) suggested that crosslinked gels could be produced through oxidation of tyrosine residues. Our work demonstrates that a composite mixture of both casein and whey proteins along with alginic acid can be crosslinked to form very stable gels with the aid of the enzyme tyrosinase. Although the mechanism of formation and stability of the gels is yet to be determined, it is known that tyrosinase catalyzes oxidation through two distinct pathways: (1) hydroxylation of monophenols and (2) dehydrogenation of *o*-diphenols (Seo et al. 2003). Our work with dairy proteins and alginic acid composites showed increased gel strength, strongly suggesting crosslinking of dairy proteins by tyrosinase.

Conclusions

Improved gelling properties can be imparted to microparticulated dairy protein products through the use of tyrosinase catalyzed crosslinking, resulting in stronger gels. There is evidence also that gel strength depended on the amount of tyrosinase. Gels increased in firmness with storage and were stable after 10 days. Competing dynamics of shear, increasing and decreasing aggregate particle sizes, intermolecular forces, and storage conditions present conditions for creating gels of varying properties.

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