6249

Rheological Properties of Thiolated and Succinylated Caseins

Gelation of casein may be improved by introduction of functional groups that are capable of forming disulfide bonds. Thiolated whole caseins were prepared with *S*-acetylmercaptosuccinic anhydride and succinylated whole caseins with succinic anhydride. Thiolated caseins formed gels at protein concentrations of 5% or more at ambient temperatures after treatment with a deacetylating agent, due to air oxidation of the sulfhydryl groups to form disulfide bonds. Gelation rate and gel strength increased with increasing concentration of casein. Gels formed at 10% protein concentration when only 2.6 lysines/mol were modified by the thiolating reagent. However, increased lysine modification was only slightly related to more rapid gelling rate or increased gel strength. Gels formed had mechanical spectra that resembled those of weak gels (gums) in terms of moduli behavior during frequency sweeps, although absolute values were greater. Succinylated caseins and native casein did not form gels under these experimental conditions.

Keywords: *Casein; gels; thiolated*

INTRODUCTION

There are several types of milk gels: rennet gels, acid gels, heat gels, and age-related gels (from concentrated milks). The primary protein constituent in all of these gels is casein. Gelation occurs when the casein micelle destabilizes and aggregates; however, caseins that are not in micellar form do not readily form gels (Brown and Ernstrom, 1988; Fox and Mulvihill, 1990).

Caseins are fairly small proteins (average molecular weight 24 000), with little secondary structure such as α-helix or β-sheet (Farrell et al., 1988), and contain, on average, only 0.4 cysteine residue/mol (Eigel et al., 1984). Therefore, casein gels are unlikely to be formed by denaturation of the secondary structure and subsequent aggregation, as frequently observed for globular proteins, or by the creation of intermolecular disulfide bonds through the oxidation of sulfhydryl groups or disulfide interchange.

Sulfhydryl (thiol) groups can be added to proteins by thiolating agents. Gels have been successfully formed with thiolated proteins. Kim et al. (1990) formed gels from β-lactoglobulin that was thiolated with *S*-acetylmercaptosuccinic anhydride (SAMSA) or with *N*-acetylhomocysteine thiolactone (*N*-AHTL). Gelation of the SAMSA-thiolated β-lactoglobulin was accomplished by the addition of Ca^{2+} and oxidation with potassium iodate. Gelation of the *N*-AHTL-thiolated β-lactoglobulin was accomplished by oxidation alone. Okumura et al. (1990) reacted α_{s1}-casein with *N*-AHTL, formed a foam of the thiolated casein, and stabilized that foam by oxidizing the sulfhydryl to disulfide bonds with potassium ferricyanide. Gelation was improved by the substitution of cysteine for a phenylalanine or for phenylalanine and an arginine in recombinant versions of β-lactoglobulin A (Lee et al., 1993). However, Murphy and Howell (1990) found that thiolation with *N*-AHTL inhibited the formation of bovine serum albumin gels and foams.

Table 1. Modification of Caseins by SAMSA and Succinic Anhydride As Measured by Decrease in Reactive Lysine and Increase in Sulfhydryl Content

mole ratio reagent:casein lysine	thiolated caseins		succinylated caseins	
	Lys[a]	SH[b]	Lys	SH
0.5:1[c]	2.6	1.40	0.8	0.03
1:1	4.59	1.98	1.62	0.02
2:1	7.29	4.32	5.40	0.04
4:1	8.78	6.02	10.12	0.03
2:1X[d]	nd[e]	7.63 ± 0.21[f]	nd	0.03 ± 0.01[g]

[a] Number of lysine residues that reacted with either SAMSA or succinic anhydride as measured by decrease in reactive lysine. [b] Number of sulfhydryl groups as measured by the method of Beveridge et al. (1974). [c] Data taken from Strange et al. (1993). [d] Data for thiolated and succinylated caseins prepared from unfreeze-dried sodium caseinate. [e] Not determined. [f] $N = 6$. [g] $N = 3$.

We prepared a series of SAMSA-thiolated caseins (Strange et al., 1993) and a similar series of succinylated caseins and described the chemical properties of these modified proteins. Caseins reacted with SAMSA have both acid and thiol components added to lysine residues, while caseins reacted with succinic anhydride have only acid components added. Therefore, differences in rheological properties of the two types of modified caseins can be attributed to the presence and reactivity of the thiol group. This paper describes the gelation of the thiolated caseins and some of the rheological properties of succinylated casein solutions and thiolated casein gels.

MATERIALS AND METHODS

Preparation and Properties of Casein and the Modified Caseins. Whole casein, 0.5:1, 1:1, 2:1, and 4:1 mol of SAMSA/mol of casein lysine (thiolated), and 0.5:1, 1:1, 2:1, and 4:1 mol of succinic anhydride/mol of casein lysine (succinylated) modified caseins were prepared as described by Strange et al. (1993). Table 1 lists the degree of lysine modification achieved as measured by reactive lysine (for both thiolated and succi-

nylated caseins) and by the presence of sulfhydryl (for thiolated casein). Differences between the number of lysine residues that have reacted with SAMSA and the number of SH groups measured are probably the result of the presence of protected (acetylated) thiols and any disulfide bonds formed. Attempts to measure disulfide were unsuccessful because of the reactivity of the exposed thiols.

Additional modified casein samples (25 g each) were prepared using 2:1 mol of SAMSA/mol of casein lysine and 2:1 mol of succinic anhydride/mol of casein lysine as described by Strange et al. (1993) with the exception that the native casein was not freeze-dried before reaction with SAMSA or succinic anhydride. These caseins will be referred to as 2:1X thiolated casein or 2:1X succinylated casein to distinguish them from the modified caseins prepared as described by Strange et al. (1993). The sulfhydryl contents of the caseins were measured using a modification (Strange et al., 1993) of a method described by Beveridge et al. (1974). This thiolated casein (2:1X) was used for experiments investigating deacetyling agents and the effect of thiolated casein concentration on gelation behavior.

Preparation of Casein Solutions. Thiolated casein solutions of various concentrations were prepared by adding sufficient casein, 1−2 mg at a time, to a weighed amount of constantly stirring water in a beaker under a continuous N_2 stream. The nitrogen is needed to exclude oxygen, preventing formation of gels during preparation of the solutions. Water that evaporated while the solutions were prepared was replaced as needed. A deacetylating agent was added to the thiolated casein solutions immediately before the samples were loaded into the rheometer. Deacetylating agents remove acetyl groups from the SAMSA-thiolated casein, exposing additional sulfhydryl groups (Klotz and Heiney, 1962). Deacetyling agents investigated were 0.1, 0.05, and 0.01 M hydroxylamine (pH 7) and 1 M imidazole. Solutions of native casein and succinylated casein were prepared similarly but, because they showed no propensity to gel, it was unnecessary to maintain the N_2 stream. No deacetyling agent was added.

Viscosity of Succinylated Caseins. The viscosities of native and succinylated casein solutions at ambient temperature (23 ± 0.5 °C) were measured using a Brookfield digital viscometer Model DV-II equipped with a UL adaptor (Brookfield Engineering Labs Inc., Stoughton, MA). Measurements were made at speeds from 0.3 to 60 rpm. Initially, the viscometer was run at the slowest speed for 2 min and percent full scale and centipoise recorded. The viscometer speed was then increased to the next higher speed, and the measurements were repeated. This procedure was continued until the maximum speed was reached and then reversed from high speed to low speed. The entire series of measurements was repeated.

Viscosity measurements were made on 10%, 7.5%, 5%, and 2.5% (w/w) triplicate native and succinylated casein solutions. Mean viscosity was calculated by averaging the viscosities recorded at various speeds when the percent full scale reading was greater than 4%.

Gelation. A Rheometrics dynamic analyzer Model 700, upgraded with Rhios software (Rheometrics Inc., Piscataway, NJ) and equipped with a 200 g·cm transducer was used for all the small-strain rheological studies. The instrument was run with the sample compartment open in a temperature- and humidity-controlled room (20−22 °C, 60−65% relative humidity). Parallel plate geometry (aluminum plates 20 mm in diameter with a 2 mm gap) was used for the gelation studies. Preliminary experiments evaluating the effects of deacetyling agents were conducted. A concentration of 0.05 M hydroxylamine was used for the gelation experiments.

Effect of Casein Concentration. Time sweep experiments were run at 1 rad/s at 3% strain for 3 h after the addition of the deacetyling agent for 12.5% and 10% 2:1X thiolated casein and for 5 h after the addition of hydroxylamine for 7.5% and 5% 2:1X thiolated casein. After the completion of the time experiments, frequency sweeps were conducted from 0.01 to 100 rad/s at 3% strain, and strain sweeps were run at 1 rad/s from 1 to 10% strain.

Table 2. Viscosity (η) in Centipoise of Native and Succinylated Caseins[a]

	concentration of casein			
mole ratio[b]	10%	7.5%	5%	2.5%
native casein	23.56[a]	8.73[a]	3.88[a]	1.94[a]
0.5:1	12.19[d]	4.77[e]	2.55[e]	1.56[d]
1:1	13.35[cd]	5.31[d]	2.69[de]	1.60[d]
2:1	13.29[cd]	5.23[d]	2.76[d]	1.66[c]
4:1	14.05[bc]	5.79[c]	3.10[c]	1.75[b]
2:1X[c]	14.80[b]	7.01[b]	3.62[b]	1.93[a]

[a] Means with the same superscript within a column are not different; $p < 0.01$ (Tukey's w procedure). [b] Modified protein made with stated mole ratio of succinic anhydride to casein lysines. [c] Modified protein made with non-freeze-dried casein.

Effect of Degree of Modification. Gelation studies on the 0.5:1, 1:1, 2:1, and 4:1 thiolated casein were conducted in duplicate at a 10% protein concentration. Time sweeps of 5 h were run on 1:1, 2:1, and 4:1 thiolated caseins and of 6 h on 0.5:1 thiolated casein. After the time experiments, frequency and strain sweeps were also completed. Test geometry and experimental parameters were the same as those used for the concentration experiment.

Statistical Analysis. ANOVA and other analyses were conducted as described in Steel and Torrie (1980).

RESULTS AND DISCUSSION

Preliminary experiments with native and succinylated casein solutions showed that they did not generate sufficient torque on the mechanical spectrometer for valid measurements when parallel plate geometry was used. Therefore, a Brookfield viscometer was used to determine the rheological effects succinylation (added acid groups) had on casein. The viscosity of native casein at concentrations 2.5−10% did not increase or decrease as shear rate increased, showing the expected Newtonian behavior (Colas et al., 1988; Konstance and Strange, 1991). This type of behavior was echoed by all five of the succinylated caseins (data not shown). Table 2 contains the mean viscosities of native and succinylated caseins at the various concentrations tested. Succinylated caseins had lower viscosities at all concentrations than native casein. The least modified of the succinylated caseins (0.5:1, 6% of lysines modified) had the lowest viscosity. Differences in the viscosities of the other succinylated caseins were inconsistent with the degree of modification. There was little difference in the viscosities of 1:1 and 2:1 succinylated caseins, although there was a large difference in the degree of modification; 12% and 40% of the lysines were modified, respectively. The 2:1X succinylated casein showed the least change in viscosity, particularly at the lower concentrations. Colas et al. (1988) showed that the major alterations in viscosity due to modification of the casein occurred at concentrations greater than about 5%. At concentrations lower than about 5%, their modified proteins had viscosities little different from that of native casein. Succinylation of other proteins showed either no effect (Franzen and Kinsella, 1976a,b) or an increase in viscosity (Habeeb et al., 1958; Beuchat, 1977; Kim and Kinsella, 1987).

The thiolated caseins had some of their sulfhydryl groups protected by acetyl groups (Klotz and Heiney, 1962). When thiolated casein solutions were prepared (preparation required several hours), they became extremely viscous and difficult to stir. However, when the solutions were kept under a N_2 stream (to exclude air) during preparation, little difficulty was encountered. Studies with the mechanical spectrophotometer showed

Table 3. Time from the Addition of 0.05 M Hydroxylamine for Thiolated Casein To Form a Gel (tan δ < 1) and Rate of Increase in G' and G'' in the First 30 min after Gelation Occurred

thiolated casein mole ratio	% protein	time to gel (min)	dyn cm^{-2} min^{-1} $\Delta G'$	$\Delta G''$
	Protein Concentration Effect			
2:1X	12.5	21	218.4	75.1
	10.0	18	43.8	22.1
	7.5	84	5.3	2.8
	5.0	256	1.2	0.6
	Modification Effect			
0.5:1	10.0	112	1.78	1.09
		66	2.08	1.01
1:1	10.0	20	1.82	0.77
		10	3.52	2.12
2:1	10.0	72	5.03	2.36
		64	1.52	0.49
4:1	10.0	18	5.56	3.03
		36	4.93	2.38

that 10% solutions of 2:1X thiolated casein treated with various deacetyling agents (0.1, 0.05, and 0.01 M hydroxylamine and 1 M imidazole) spontaneously formed gels when exposed to air. Solutions (10%) deacetylated with 0.01 M hydroxylamine or 1 M imidazole took 3.25 and 5.75 h to gel, respectively, while solutions containing 0.1 and 0.05 M hydroxylamine gelled after 16 and 18 min, respectively. Gelation was assumed to have occurred when the tan δ of the solutions was less than 1 [storage or elastic modulus (G') exceeded the loss or viscous modulus (G'') (Ross-Murphy, 1991)]. Deacetylation with hydroxylamine generates carbon dioxide by the Lossen rearrangement of hydroxamic acid. Gelation trapped some of this carbon dioxide. However, use of the 0.05 M hydroxylamine provided both satisfactory speed for gelation and formation of fewer bubbles.

The gelation of 12.5%, 10%, 7.5%, and 5% solutions of 2:1X thiolated casein treated with 0.05 M hydroxylamine was monitored at 1 rad/s and 3% strain. The time taken for a casein gel to form (tan δ < 1) and the rate at which storage and loss moduli increased in the first 30 min after gelation are reported in Table 3. It obviously took much less time for 12.5% and 10% solutions of casein to gel than for 7.5% and 5% solutions, and the rate at which the moduli increased was much slower for the less concentrated solutions. In all cases, the elastic modulus increased at a faster rate than did the viscous modulus, another indicator of gelation (Ross-Murphy, 1991). The time sweep for 10% 2:1X thiolated casein treated with 0.05 M hydroxylamine is shown in Figure 1. The time sweeps for the other concentrations of casein have different shapes but all share several major features. Before gelation occurred, the G' and G'' data were unreliable because the torque generated by the solutions was below the sensitivity of the transducer. Sufficient torque for valid measurements was obtained about the same time as gelation occurred. After gelation, the G' was always greater than G'', G' increased at a faster rate than G'', and the moduli were still increasing at the end of the time sweeps. This continuing increase was probably due to a combination of evaporation of the water from the gel and continuing oxidation of the sulfhydryl groups.

Frequency sweeps of gels of the four 2:1X thiolated casein concentrations were made 3.5 h after the addition of hydroxylamine for 12.5% and 10% gels and 5 h after addition for 7.5% and 5% gels. Figure 2 shows the effect of concentration on G', G'', and tan δ as a function of frequency. G' and G'' increased slightly as frequency

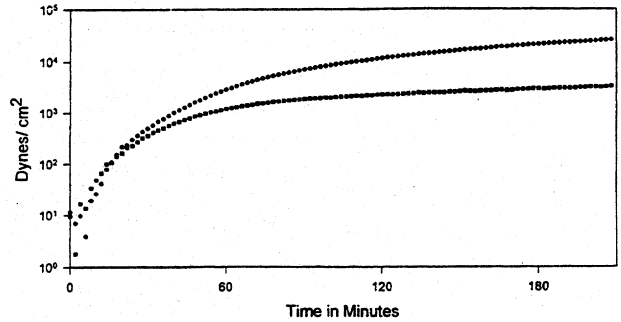

Figure 1. Time sweep at 1 rad/s and 3% strain for 10% (w/w) 2:1X thiolated casein treated with 0.05 M hydroxylamine: (●) G'; (■) G''.

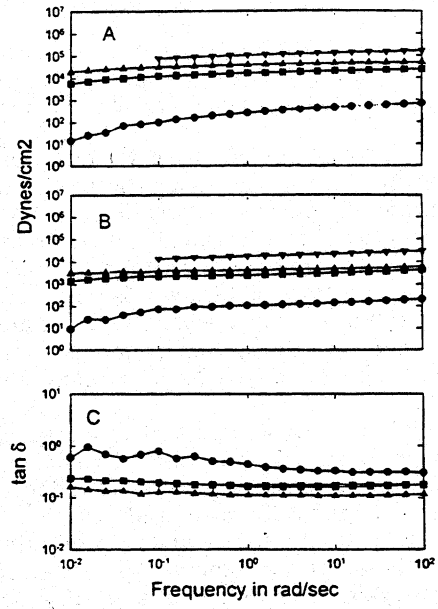

Figure 2. (A) G' in dyn/cm^2 for (▼) 12.5%, (▲) 10%, (■) 7.5%, and (●) 5% w/w 2:1X thiolated casein versus frequency in rad/s 3 h after the addition of 0.05 M hydroxylamine for 12.5% and 10% solutions and 5 h after the addition of 0.05 M hydroxylamine for 7.5% and 5% solutions. (B) G'' in dyn/cm^2 for (▼) 12.5%, (▲) 10%, (■) 7.5%, and (●) 5% w/w 2:1X thiolated casein versus frequency in rad/s 3 h after the addition of 0.05 M hydroxylamine for 12.5% and 10% solutions and 5 h after the addition of 0.05 M hydroxylamine for 7.5% and 5% solutions. (C) Tan δ (G''/G') for (▼) 12.5%, (▲) 10%, (■) 7.5%, and (●) 5% w/w 2:1X thiolated casein versus frequency in rad/s 3 h after the addition of 0.05 M hydroxylamine for 12.5% and 10% solutions and 5 h after the addition of 0.05 M hydroxylamine for 7.5% and 5% solutions.

increased, and tan δ was independent of frequency. G'' was parallel to G', and the force developed was slightly less than 1 log smaller. Strain sweeps of the gels showed linear behavior for the moduli between 0% and 10% strain. Attempts to measure the moduli at higher strains were unsuccessful because the torque exceeded the transducer capacity at 16% strain for 12.5% 2:1X thiolated casein.

G' and G'' of the gels were larger as the concentration of the casein increased, but all gels had similar properties. The frequency sweeps showed that the storage and loss moduli behaved in a parallel manner and increased slightly as frequency increased. The tan δ values achieved by the gels were not related to the concentration of the casein and, in all cases, were less than 1 but greater than 0.1. The strain sweeps showed extensive linear behavior. This type of mechanical spectrum is consistent with a weak gel of the type typically found

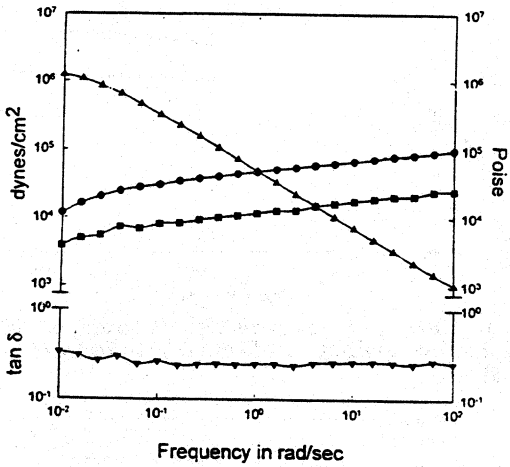

Figure 3. (●) G', (■) G'', (▲) η^*, and (▼) tan δ for 4:1 thiolated casein 5 h after the addition of 0.05 M hydroxylamine.

for gums such as xanthan (Haque et al., 1993) and guar (Carnali, 1992) with the exceptions that the G' and G'' are much larger (Haque et al., 1993; Carnali, 1992) and the linear behavior of the tan δ with frequency is consistent with a strong gel (Clark and Ross-Murphy, 1987). The linear strain (γ) behavior is consistent with a covalent cross-linked system which is related to rubber elasticity rather than the typical biopolymer gels with a much smaller linear range (Clark and Ross-Murphy, 1987).

The gelation of 10% solutions of thiolated caseins prepared with 0.5:1, 1:1, 2:1, and 4:1 mole ratios of SAMSA to casein lysines also was monitored for 5 h for 1:1, 2:1, and 4:1 thiolated casein and for 6 h for 0.5:1 thiolated casein at 3% strain and 1 rad/s after the addition of 0.05 M hydroxylamine. All of the thiolated caseins formed gels. However, the results after 5 h were extremely variable; the duplicates of only the 4:1 thiolated casein approached one another. ANOVA of the rate at which G' increased after gelation (Table 3) shows no significant differences among the thiolated casein samples. G'', G', and tan δ of one of the gels formed by 4:1 thiolated casein (5 h after the addition of hydroxylamine) as a function of frequency are shown in Figure 3. This mechanical spectrum is typical of the gels formed by the modified caseins; the differences are in the amount of force generated, not in the shape of the response. The slight curvature in G' and G'' at extremely low frequencies may due to the length of time needed for accurate measurment at these very low frequencies (up to 20 min) compared to that (1–2 s) needed at the higher frequencies. No pattern in the size of the G' and G'' moduli that developed after 5 (or 6) h was evident as related to the degree of substitution. However, if the elastic moduli (G') were compared 3 h after the addition of hydroxylamine, the average G' developed increased as the number of modified lysines increased. Although the tan δ at 3 h after the addition of hydroxylamine was higher for 0.5:1 than for 4:1 thiolated casein, the tan δ of caseins with intermediate levels of modification were inconsistant with level of modification.

Gels were formed at all concentrations of the thiolated caseins tested. No experiments were performed at concentrations less than 5% because of the limited sensitivity of the measuring system. All gels formed were exceedingly tough and difficult to remove from the parallel plates of the spectrometer. To achieve greater sensitivity, Couette geometry would have been needed and the possibility existed for achieving permanent bonding between the cup and the bob.

CONCLUSIONS

Gels were formed by the thiolated caseins at protein concentrations as low as 5% at ambient temperatures (20–22 °C) due to air oxidation of the sulfhydryl groups to form disulfide bonds after treatment with deacetyling agents. Succinylated caseins and native casein did not form gels under these conditions. In fact, succinylation decreased the viscosity of casein. The rheological properties of thiolated casein gels depended on the concentration of the casein and to a lesser extent on the number of sulfhydryl groups present. Failure of other researchers to form gels of proteins modified by SAMSA (Murphy and Howell, 1990) or to form gels successfully at only high levels of modification (Kim et al., 1990) and in the presence of calcium ion is probably due to deacetylation at low protein concentration encouraging the formation of intramolecular disulfide bonds or polymers instead of the extended structure of a gel. Protection from oxidation by dissolving the thiolated casein under N_2 contributed to the availability of SH groups to participate in gel formation. Additional experiments have shown that adding potassium iodate (an oxidizing agent) at the same time as NaOH (a deacetylating agent) to a 10% solution of 1:1 thiolated casein resulted in rapid gel formation.

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