# Thermal Gelation of the 12S Canola Globulin

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**The nature of intermolecular forces responsible for thermal gelation of the 12S canola globulin was determined by preparing gels under a variety of environmental influences. The effects of pH, sodium salts and denaturing agents were evaluated by differential scanning calorimetry, small amplitude oscillatory rheology and transmission light microscopy. Gels prepared with 6% protein at alkaline pH values were superior to gels prepared under acidic conditions. Sodium salts, which promoted protein stability, had an adverse effect on gelation. The addition of guanidine hydrochloride and dithiothreitol to protein dispersions prior to heating produced inferior gels. Hydrophobic forces and electrostatic interactions were responsible for the establishment of canola gel networks. Gel stabilization and strengthening were attributed to disulfide bonding, electrostatic interactions and hydrogen bonding.**

KEY WORDS: Canola, dynamic rheology, gelation, 12S globulin, microstructure, protein.

Canola meal has been traditionally marketed as a feed supplement for livestock and poultry, yet it represents a potential source of protein for human nutrition. Canola protein has a well-balanced amino acid profile (1–4) and a high protein efficiency ratio (5,6). Isolation of canola protein has been the emphasis of many investigations. One such method, which involves a mild salt extraction and precipitation by dilution, has produced a protein isolate with low residual levels of antinutritional substances and little change in protein conformation (7,8). Utilization of an isolate of this type requires further knowledge of its functional behavior.

One functional property that can be of merit in the utilization of a plant protein is the ability to form a gel. Although gelation mechanisms of soy proteins and other related plant proteins have received much attention, insight into the gelation of canola protein is limited. The ability of the 12S canola protein to form gels was first demonstrated in 1978 (9). It was concluded that gelation occurred through a complex phenomenon involving both covalent and noncovalent forces, though the roles of ionic and disulfide bonding were considered minor. Since then, several studies have found the gelation characteristics of canola protein to be similar to those of soy protein (10,11).

Not all studies have confirmed the gelling ability of canola protein. Working with a hexametaphosphate-extracted protein and heating to 80°C for 30 min, Thompson *et al.* (12) demonstrated an increase in viscosity, but were unable to form gels. Work done by Paulson and Tung (13), which involved heating to 72°C, produced canola protein gels only at high pH values or when the protein had been succinylated. The properties of the gels were dependent on degree of succinylation, NaCl concentration and pH. The major forces responsible for gelation in this study were believed to be hydrophobic interactions and hydrogen bonding.

In view of the reported denaturation temperature of 88°C for the 12S canola protein at pH 6 (8), it is questionable whether the heating temperatures in some of these studies were sufficient to denature the protein, a necessary step in the gelation process. In addition, most researchers have relied on static methods of measuring final gel structure rather than dynamic methods.

In the present study, the gelation properties of canola protein isolated under mild conditions have been examined to investigate the intermolecular forces responsible for the gelation of the 12S canola protein. Variations in pH, salt type and the presence of denaturing agents have been used to study these forces. Thermal denaturation temperatures have been determined for each environment to ensure that the heating temperature was sufficient to promote gelation. In addition to characterizing the gels by small-amplitude oscillatory rheology and light microscopy, changes in rheological properties during the heating and cooling phases of gel formation have been analyzed to establish the roles of various molecular forces in the formation of canola protein gels.

## EXPERIMENTAL PROCEDURES

*Source of material.* A commercial canola meal (*Brassica campestris* var. Tobin), defatted by a prepress solvent technique, was used. The 12S globulin was isolated by means of a modified version of the protein micellar mass technique described by Murray and co-workers (14). To extract the protein, finely ground canola meal was mixed with 0.1 M NaCl (pH 6.2) at a ratio of 1:10 and stirred for 1 h at room temperature. The mixture was centrifuged at 958 × *g* for 30 min at 10°C, and the supernatant was concentrated to approximately one-third its original volume in an ultrafiltration unit with a molecular weight cut-off of 10,000 daltons and operated at 3.8–4.1 bar. To precipitate the protein, the retentate was added to cold, distilled water at a ratio of 1:6 and allowed to stand at 4°C for at least 16 h. The isolated protein was collected by centrifuging the suspension at 16300 × *g* for 30 min at 10°C. The protein pellet was frozen at −32°C and subsequently freeze-dried.

Unless stated otherwise, all chemicals used in this study were Certified A.C.S. grade and supplied by Fisher Scientific Co. (Nepean, Ontario, Canada).

*Sample preparation.* All samples were prepared with 6% protein at pH 9, except when the effects of pH were being assessed. For the pH series, samples were prepared with 0.1M $Na_2B_4O_7$ (pH 9.0), and the pH was adjusted to 4, 5, 6, 7, 8, 9, 10 and 11 with either 1M HCl or 1M NaOH. The borate buffer was used to prepare solutions containing 0.0–3.0M guanidine hydrochloride (electrophoresis grade; Fisher Scientific Co.) and 0.0–0.15M dithiothreitol (Sigma Chemical Co., St. Louis, MO). For the salt series, solutions of $Na_2SO_4$, $NaC_2H_3O_2$, NaCl and NaSCN were prepared with distilled water rather than with the buffer. The pH was adjusted to pH 9 with 1M NaOH.

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On any given day, the effects of one environmental condition were assessed in duplicate. For each duplicate, a 5-mL sample was prepared in a capped cylindrical glass vial (17 mm i.d., 15-mL capacity). The protein was solubilized/dispersed in the selected solvent. The pH was adjusted if necessary. A 0.5-mL aliquot from each sample was transferred to a capped vial for analysis by differential scanning calorimeter (DSC). Approximately 1 mL of sample was removed for rheological characterization, and the remaining 3.5 mL was used for microscopic examination.

*Calorimetry.* The thermal properties of the 12S globulin were evaluated to assess the effect of imposed environment on protein conformation prior to heat-induced gelation. DSC was performed with a Dupont 9900 Thermal Analyzer (TA Instruments, New Castle, DE) with a 910 Cell Base. The sample was heated over a temperature range of 35–110°C at a rate of 10°C $min^{-1}$ in a standard DSC cell that had been calibrated with both indium and sapphire standards. Aliquots of sample measuring 10–15 μL were analyzed with an empty pan as reference. Each sample was analyzed in triplicate. Both the thermal denaturation point (Td), which was measured at the point of maximum heat flow, and the enthalpy of denaturation (ΔH) were calculated.

*Rheology.* A Bohlin VOR rheometer (Bohlin Reologi, Inc., Edison, NJ) operated in the small-amplitude oscillatory mode, was used to follow protein gel formation during heating and cooling and to characterize the rheological properties of resulting gels. The rheometer was equipped with 30-mm parallel plate geometry, and sensitivity was established based on a torque barr calibrated to 93.2 gcm. Input strain amplitude for dynamic analysis was 0.02, a value found to be in the linear viscoelastic region during preliminary experimentation. This strain was used for all rheological measurements.

Approximately 1 mL of protein dispersion was applied to the lower platen of the parallel-plate geometry so that a gap of 1.0 mm was realized when the upper platen was lowered. A 16-cm strip of masking tape was wrapped around the circumference of the cylinder supporting the lower platen to from a well to prevent sample drying during heating. Paraffin oil (Saybolt viscosity 125/135, Fisher Scientific Co.) was added to the constructed well until the upper platen was covered. Once in place, samples were subjected successively to a heating phase, a cooling phase and a frequency sweep. Sample temperature was controlled by a programmable water bath.

Samples were heated and then cooled over a temperature range of 25–90°C at a rate of 2°C $min^{-1}$. A frequency of 0.10 Hz was used for the thermal scans. Rheological data were collected every 2.0 min with a thermal equilibrium time of 10 s. At the end of each phase, the final temperature was held for 3.0 min. Frequency sweeps of the final product were conducted over a range of 0.01–10 Hz at 25°C. Rheological properties were expressed in terms of storage modulus (G′) and loss modulus (G″). The loss tangent was calculated manually as tan $\delta = G''/G'$.

*Microscopy.* Before samples were prepared for microscopic examination, the pH of the dispersions was verified and readjusted if necessary. Glass vials containing 3.5 mL of dispersed protein sample were capped with pieces of aluminum foil. Samples were heated over a range of 25–90°C at a rate of 2°C $min^{-1}$. At the end of the heating cycle, samples were maintained at 90°C for 3.0 min. After 3.0 min, the vials were placed in an ice bath until the samples reached room temperature.

Gels were removed from the vials and sliced to a thickness of approximately 3 mm, discarding the outer edges of the slices. The gel cores were mounted on specimen holders with a water-soluble glycol-resin embedding compound (Tissue-Tek O.C.T. Compound 4583; Miles Scientific, Naperville, IL). The samples were quickly frozen by placing the specimen holders in the heat sink of a cryomicrotome (Cryo-Cut II, Model 851C; American Optical, Buffalo, NY). The temperature of the microtome cabinet was maintained at −25 ± 5°C. Frozen gel segments were sectioned to a thickness of 7 μm. Sections were mounted directly onto glass slides by adhesion by holding a warm slide against the cut specimen. Specimens were neither stained nor covered with a cover slip.

A Zeiss Universal Research Microscope (Carl Zeiss Canada, Don Mills, Ontario, Canada) was used to examine gel networks. Specimens were observed by brightfield microscopy, with a blue filter in place to improve the contrast between the image and the background. Photomicrographs were taken with either a C35M or a MC100 Zeiss camera on Kodak Ektachrome 160 film.

*Statistical analysis.* All experimental environments were examined in duplicate. Values obtained from DSC and rheological studies were averaged and reported as means. Statistical differences within each series of tests were determined by an analysis of variance in conjunction with a Duncan's multiple range test (15). This was done on an IBM computer with a Number Crunching Statistical System software package.

## RESULTS AND DISCUSSION

*Effect of pH.* The effect of pH on canola protein structure prior to gelation was reflected in Td and ΔH values (Table 1). The pH had a significant impact on protein conformation; especially at pH 11 and at pH values below 7, both Td and ΔH values decreased significantly. The Td value observed in this study at pH 6 (≈8.0°C) was lower

TABLE 1

**Effect of pH on the Thermal Denaturation of 6% Canola Protein and Rheological Properties of 6% Canola Protein Gels at 1 Hz[a]**

| pH | Td[b] (°C) | ΔH[c] (J/g protein) | G′[d] (Pa) | Tan δ[e] |
|---|---|---|---|---|
| 4 | — | $0^{f}$ | $10^{f}$ | $0.202^{f,g}$ |
| 5 | $75.42^{f}$ | $13.20^{g}$ | $214^{f,g}$ | $0.187^{f,g}$ |
| 6 | $79.97^{g,h}$ | $15.44^{h}$ | $169^{f,g}$ | $0.204^{g}$ |
| 7 | $80.92^{h,i}$ | $17.03^{i,j}$ | $506^{g}$ | $0.198^{f,g}$ |
| 8 | $80.24^{h,i}$ | $16.82^{h,i}$ | $2560^{j}$ | $0.157^{f,h}$ |
| 9 | $81.01^{h,i}$ | $18.44^{j}$ | $1660^{i}$ | $0.106^{i,j}$ |
| 10 | $81.26^{i}$ | $16.28^{h,i}$ | $1040^{h}$ | $0.088^{j}$ |
| 11 | $79.03^{g}$ | $12.58^{g}$ | $84^{f}$ | $0.138^{h,i}$ |

[a]Column values followed by the same letter are not significantly different ($P < 0.05$). Td, thermal denaturation point; ΔH, enthalpy of denaturation.
[b]SE = 0.37.
[c]SE = 0.52.
[d]SE = 110.
[e]SE = 0.013.

than the 88°C reported previously for a similar heating rate (8). This difference may be due to differences in the material examined (*B. campestris* in this study and *B. napus* in the earlier work), or differences in the processing history, such as differing levels of protein denaturation during oil removal.

Theoretically, conformational changes of proteins caused by pH are due to ionization of amino acid side chains. At its isoelectric point (IEP), a protein carries no net charge and exists in its most stable conformation. As the pH moves away from the IEP, the magnitude of the net charge increases. This increase in repulsive charges causes proteins to unfold and reduces the potential for electrostatic interactions among neighboring polypeptides. As a result, protein functionality can be affected. With ovalbumin (from egg) and vicilin (from fababean), pH affects the overall charge repulsion and, consequently, the interactions between proteins that contributed to the characteristics of the resulting gels (16).

Although the potential number of negatively charged residues at pH values above canola's IEP is larger than the potential number of positively charged residues at pH values below its IEP (17), pH exhibited a greater effect on Td and ΔH values in the acid range. Similar results have been reported for fababean protein (18). This behavior has been related to the fact that ΔH values are a sum of both endothermic and exothermic reactons, and that conformational rearrangement may be more involved than can be detected through DSC measurements.

All samples, except those prepared at pH extremes, displayed structure development upon heating, as reflected by increased G′ values. Samples prepared at pH 5, 6 and 7 exhibited a rise in the G′ modulus at or slightly below their respective thermal denaturation temperatures. This would suggest that ionization and subsequent conformational changes induced by the acidic environments promoted protein-protein interactions prior to significant thermal denaturation. As discussed above, pH exerted its greatest effect on protein conformation at pH values below 7. At pH 4, where the protein was completely denatured by the acidic condition, the overall net repulsive charge was evidently too high for structure formation.

At pH values above 7, the samples that demonstrated increased G′ values did so above their respective thermal denaturation points. For these samples, it was apparent that thermal denaturation was a prerequisite for the formation of rheologically significant structures. Again, the excessive repulsive charge on samples prepared at pH 11 was presumed to be responsible for the lack of structure formation during heating.

Representative cooling curves are shown in Figure 1. The G′ values for all samples increased gradually, albeit ever so slightly, for samples prepared at pH 4 and 11. The shape of the cooling curves varied. These results indicated that the mechanism by which gelation occurs is influenced by pH. At pH 4 and 11, the potential for electrostatic interactions and subsequent gel formation appears to be limited by an overall excess of repulsive charges on the polypeptide chains. At pH 5 and 6, it seemed as if aggregation reactions are favored during the heating cycle. As a result, significant structure development is prevented during the cooling phase. At pH 7, 8, 9 and 10, various cooling patterns were observed. Given the similarity of the thermal analysis results, it would seem that this is due to the degree of ionization and the potential for electrostatic interactions rather than to the extent of conformational change. However, it is possible that conformation changes resulting from different pH environments could have impacted the mode of gelation.

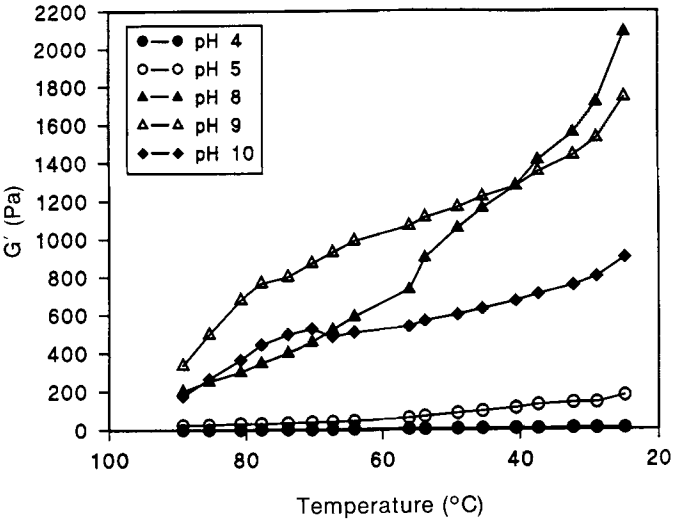


**FIG. 1. Effect of pH on G′ as a function of temperature during cooling of 6% canola protein. Curves not shown for pH 6 and 7 were similar to that for pH 5, and the curve for pH 11 was similar to that for pH 4.**

The G′ and tan δ values for the final gel structures are shown in Table 1. Maximum G′ values were obtained at pH 8. Below and above this pH, the decrease in G′ modulus was significant. The G′ modulus is thought to relate to the number of crosslinks within a gel system A(6,7). At pH 8, close to the IEP of canola protein, attractive forces are expected to be high, causing random aggregation of molecules. In some systems, clumps of aggregates have been shown to yield high G′ values due to the relative closeness of neighboring molecules (8). As pH moves away from the IEP, protein–solvent reactions are favored over protein–protein reactions, resulting in fewer crosslinks and, hence, lower G′ values.

The lowest tan δ values were recorded for the gels prepared at pH 10. The increased electrostatic repulsion at this pH must minimize random aggregation and reduce the degree of interaction among polypeptides in the system. The development of superior network structures at high pH values has been attributed to the extension of polypeptide chains and the creation of homogeneous structures (13). In comparison, gels prepared at lower pH values are heterogeneous in nature. These heterogeneous networks are composed of compact molecules, which aggregate to form regions of high and low protein concentrations. As a result, regions with low protein concentrations act as weak points, causing a drop in overall gel strength and higher tan δ values. At pH extremes, such as pH 11, the dominant net repulsive charge favors protein-solvent reactions over protein-protein reactions and yields relatively weak gels.

Photomicrographs for the pH series of gels are given in Figure 2. No photomicrographs were obtained for samples prepared at pH 4 because self-supporting gels could not be obtained. The photomicrographs supported the rheological properties that characterized the final gels.

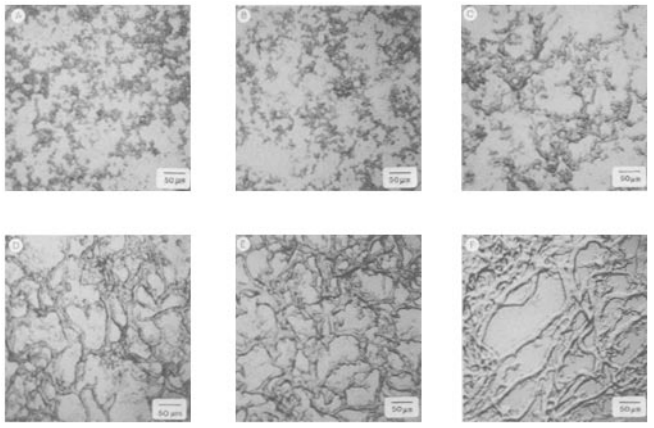

**FIG. 2. Photomicrographs of 6% canola protein gels prepared in various pH environments. A, pH 5; B, pH 7; C, pH 8; D, pH 9; E, pH 10; and F, pH 11. The photomicrograph for pH 6 (not shown) was similar to those at pH 5 and 7.**

The G′ modulus tended to relate to the compactness of the structures, whereas tan $\delta$ values best reflected the actual arrangement of the networks.

*Effect of sodium salts.* The Td and ΔH values for canola protein exposed to various sodium salts are presented in Table 2. Salt type significantly affected the thermal denaturation properties of the 12S canola globulin. Samples prepared in the presence of salts displayd higher thermal denaturation temperatures than the control containing no salt (Td = 81.0°C). The increase in thermal denaturation temperatures with the inclusion of low salt concentrations can be attributed to nonspecific ion effects on electrostatic interactions between charged groups on the protein. This resulted in stabilization of the globulin against thermal denaturation by all anions. Significant differences in Td values among the four salts indicate that anions exert ion-specific stabilizing or destabilizing effects at unusually low concentrations. Similar results have been reported for the 11S soy globulin (19).

Despite the increase in thermal denaturation temperatures, the ΔH values for the canola protein in the 0.1M salt series were slightly lower than that of the 6% protein gel prepared at pH 9 without salt (ΔH = 18.44 J/g). Damodaran (19) offered two possible explanations for this phenomenon. Although higher Td values reflect increased resistance to thermal denaturation, the tertiary and quaternary structures of the stabilized protein may not be the same as that of the native molecule. Consequently, a lower ΔH can be obtained. Alternatively, the apparent decrease in ΔH values may be due to aggregation of denatured protein. The exothermic heat effect of such an aggregation process may partly offset the endothermic heat flow measured by DSC.

At higher concentrations, neutral salts are known to have ion-specific effects on hydrophobic interactions in addition to nonspecific charge neutralization effects. These ion-specific effects on hydrophobic interactions are believed to arise from perturbations in bulk water structure, which affect protein-solvent and protein-protein interactions (20). In this respect, $Na_2SO_4$ acts as a promoter of hydrophobic interactions, whereas NaSCN acts as a protein destabilizer, the result of which is binding of NaSCN to the protein and creating a net charge repulsion. In the present study, the degree to which the thermal denaturation temperature was affected by the anions depended on the position of the anions in the following sequence: $SO_4^= > C_2H_3O_2^- > Cl^- > SCN^-$. Although this effect is more commonly observed at higher salt concentrations (0.5M or more), this phenomenon was evident at 0.1M salt concentration in this study.

Despite the fact that $SCN^-$ is regarded as a destabilizing anion, its Td value was relatively high. Similar results have been reported for the 11S soy globulin (19) where, in the presence of destabilizing anions, Td values rose until the anion concentration reached 0.5M. Consequently, the destabilization effect became more pronounced as concentrations were increased to 2–3M.

The rheological data for the 12S canola protein in the presence of these sodium salts showed structure development for all four treatments. However, the extent to which structure developed among the four samples was much less than that of samples prepared without salt at pH 9, as seen in the pH series. During the heating phase, the $SCN^-$ anion showed the greatest increase in G′ values, followed by $Cl^-$, $C_2H_3O_2^-$ and $SO_4^=$, respectively. This pattern prevailed during the cooling regime as well (Fig. 3). The $SCN^-$ ion exhibited the greatest increase in the G′ modulus, whereas the $SO_4^=$ ion exhibited the smallest increase. The shapes of the cooling curves were similar for all treatments, suggesting that all underwent the same gelation mechanism. A slight rise in the rate of structure formation was noted at approximately 56°C.

Rheological properties of the final gels are shown in Table 2. Significant differences in gel characteristics were observed among the four treatments. It appears as if the ability of the 12S canola globulin to form gels in the presence of 0.1M sodium salts is related to the thermal denaturation temperature of the protein when exposed to each of the four anions. As the thermal denaturation temperature increases, structure development is delayed, and ensuing gel networks are inferior in overall strength and structure. Although electrostatic interactions were thought to be the principal noncovalent force at work at low ionic strengths, the rheological data support the possibility of lytotropic effects on protein structure development at the 0.1M salt level. This may explain the favorable characteristics of gels prepared in the presence of $SCN^-$. As a destabilizing anion, $SCN^-$ has the added

**TABLE 2**

**Effect of 0.1M Sodium Salts on the Thermal Denaturation of 6% Canola Protein at pH 9 and Rheological Properties of 6% Canola Protein Gels (pH 9) at 1 Hz[a]**

| Salt | Td[b] (°C) | ΔH[c] (J/g protein) | G′[d] (Pa) | Tan $\delta$[e] |
|---|---|---|---|---|
| Sodium sulfate | 87.40[f] | 16.67[f] | 78[f] | 0.127[f] |
| Sodium acetate | 87.02[f] | 17.51[g,h] | 138[f] | 0.124[f] |
| Sodium chloride | 85.78[g] | 17.67[h] | 210[g] | 0.113[g] |
| Sodium thiocyanate | 85.75[g] | 16.68[f,g] | 426[h] | 0.094[h] |

[a]Column values followed by the same letter are not significantly different ($P < 0.05$). Abbreviations as in Table 1.
[b]SE = 0.13.
[c]SE = 0.28.
[d]SE = 18.
[e]SE = 0.002.

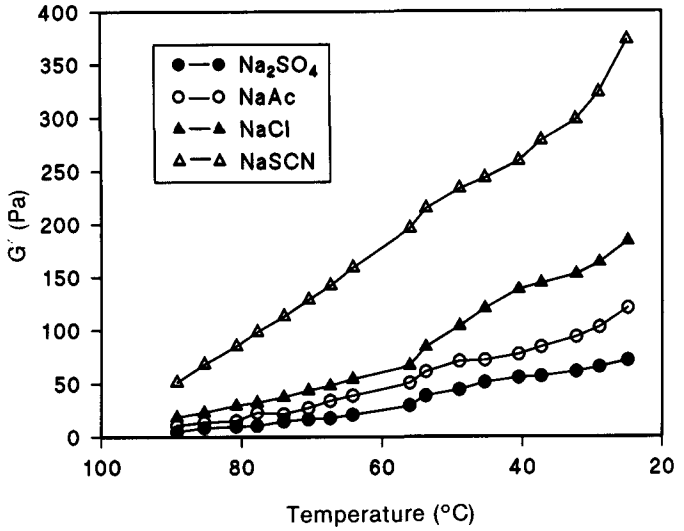


**FIG. 3. Effect of 0.1M sodium salts on G′ as a function of temperature during cooling of 6% canola protein at pH 9.**

capability of binding to proteins. This not only changes the charge profile of the protein but, by creating an excessive negative charge, $SCN^-$ helps to unfold the globulin, exposing formerly buried functional groups and making them available for network crosslinking. On the other hand, $SO_4^=$ anions are known stabilizers of protein molecules. As a result, the associating structures within the network remain in a more globular form in which nonpolar groups are buried and, therefore, hydrophobic interactions among neighboring polypeptides are minimized. Thus, it was evident that hydrophobic interactions as well as electrostatic interactions contribute to the gel formation of the 12S canola protein.

The photomicrographs (not shown) provided substantial support for the DSC and rheological findings. The structures of the four salt-prepared gels never developed to the extent of gels prepared under the same conditions without salt. The $SCN^-$-treated gels consist of loosely connected strands of extended protein, whereas the strands of $Cl^-$ samples are less well-defined, and the network remains loose and disconnected. Although gels prepared with $C_2H_3O_2^-$ appeared to be more dense, it was evident that strand formation was underdeveloped. In the case of $SO_4^=$ samples, no protein strands were noted; only an arrangement of clumped protein was apparent as indicated by lower G′ and higher tan $\delta$ values in the rheological data.

*Effects of guanidine hydrochloride (GuanHCl).* Both thermal denaturation properties indicated that GuanHCl has a significant impact on protein conformation (Table 3). This effect became more apparent as the GuanHCl concentration was increased from 0.1 to 3.0M. At relatively low concentrations, GuanHCl was capable of exerting a salt-induced effect on proteins. As seen in the sodium salt series, a higher thermal denaturation temperature and lower enthalpy of denaturation were noted with the 0.1M GuanHCl sample as compared with the control. This increase in the thermal denaturation temperature may be due to nonspecific ion effects caused by the guanidine salt. Similarly, the decrease in the ΔH value for the same sample may be due to the same factors responsible for similar

**TABLE 3**

**Effect of Guanidine Hydrochloride (GuanHCl) Concentration on the Thermal Denaturation of 6% Canola Protein at pH 9 and Rheological Properties of 6% Canola Protein Gels (pH 9) at 1 Hz[a]**

| GuanHCl concentration (M) | Td[b] (°C) | ΔH[c] (J/g protein) | G′[d] (Pa) | Tan δ[e] |
|---|---|---|---|---|
| 0 | 81.01[f] | 18.44[f] | 1660[f] | 0.106[f] |
| 0.1 | 86.54[g] | 15.38[g] | 956[g] | 0.126[f] |
| 1.0 | 85.11[h] | 10.20[h] | 393[h] | 0.118[f] |
| 3.0 | — | 0[i] | 1[i] | 1.058[g] |

[a]Column values followed by the same letter are not significantly different ($P < 0.05$). Abbreviations as in Table 1.
[b]SE = 0.21.
[c]SE = 0.58.
[d]SE = 48.
[e]SE = 0.159.

behavior with the sodium salts; that is, the structure of the stabilized protein may not be the same as the native molecule, and/or an exothermic reaction caused by protein aggregation during thermal analysis may create a net decrease in endothermic heat flow (19).

Samples prepared with 1.0M GuanHCl gave lower Td and ΔH values than samples prepared with 0.1M GuanHCl; however, the Td of the 1.0M GuanHCl samples was higher than the Td of the control. At this concentration, it appeared as if GuanHCl exerted denaturing effects. As a competitive hydrogen bond former, GuanHCl is capable of strong interactions with water, which alter the structure of the aqueous phase around the protein molecule and increase the solubility of hydrophobic amino acids. As a result, GuanHCl can disrupt both hydrogen bonds and hydrophobic interactions involved in the maintenance of protein structure. Both factors could have contributed to conformational changes. The denaturing effects of GuanHCl were more pronounced at the 3.0M concentration, indicating destabilization and complete unfolding.

The effect of increasing GuanHCl concentration on canola protein gelation was evident during all stages of the rheological study. At the end of the heating phase, the control sample gave the greatest G′ value, whereas the 0.1 and 1.0M samples gave progressively lower values. Lower G′ values for the 0.1M GuanHCl treatment were attributed to a salt effect similar to that seen with the sodium salts. At 1.0M GuanHCl, conformational changes may also have impeded structure development. The samples prepared in the presence of 3.0M GuanHCl did not show any evidence of significant rheological structures. This indicates that a high concentration of GuanHCl is capable of interfering with the intermolecular forces responsible for structure development during the early stages of gelation.

Data collected during the cooling phase provided more support for this theory. Both the shape and the slope of all four cooling curves were distinctly different (Fig. 4). The shape of three of the four cooling curves were biphasic in nature. Of particular interest was the temperature at which the rate of G′ increase changed. For the control, the rate changed at approximately 80°C. For the 0.1 and 1.0M GuanHCl samples, the rate change occurred at approximately 66 and 55°C, respectively. With 3.0M GuanHCl, G′ values remained at the baseline during the entire

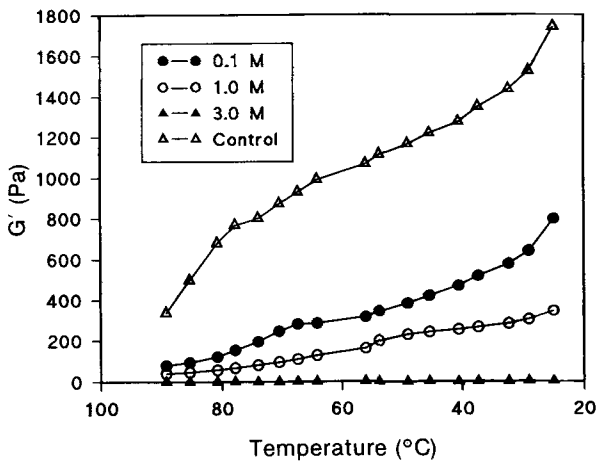


**FIG. 4. Effect of guanidine hydrochloride concentration on G′ as a function of temperature during cooling of 6% canola protein at pH 9.**

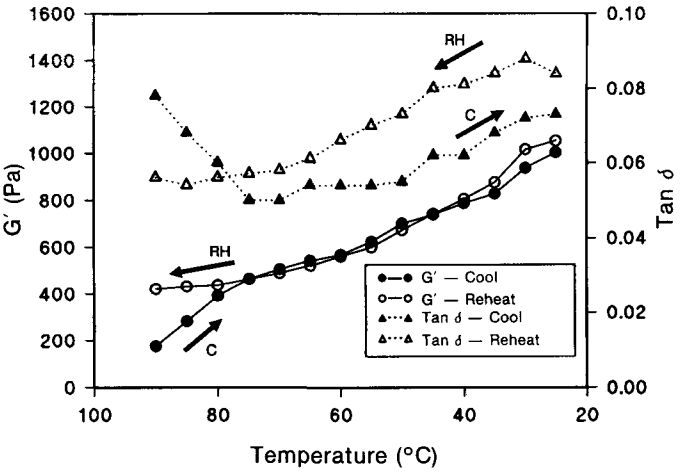


**FIG. 5. Effect of reheating on G′ and tan δ values of 6% canola protein gels prepared at pH 9.**

cooling period. From these results, it is evident that higher concentrations of GuanHCl extend the initial stage of structure development. Given the effect of GuanHCl on hydrophobic interactions and the fact that these interactions are favored at higher temperatures, it is possible that hydrophobic forces play a major role in gel formation during both heating and initial cooling phases.

With regard to the slopes of the cooling curves, the rate at which G′ modulus increased was inversely related to the GuanHCl concentration. As the concentration of GuanHCl increased, the rate at which G′ values increased declined to the point where no structure formation was observed at 3.0M GuanHCl. As mentioned above, the effect of GuanHCl on hydrophobic forces may have reduced the rate at which G′ increased during the initial cooling period. During the latter phase of cooling, the reduction in rate of G′ increase may have been due to the effect of GuanHCl on hydrogen bonds as these bonds are favored at lower temperatures.

Overall, GuanHCl may have prevented the formation of hydrophobic interactions, which, in turn, prolonged the initial stage of gel formation and limited the extent to which structure developed. In addition, GuanHCl concentration influenced the extent to which the established structure was strengthened, possibly through hydrogen bonding during the latter phase of cooling. It has been postulated previously that hydrophobic interactions and hydrogen bonds are involved in the formation and stability of gels prepared from succinylated canola protein (13). Further evidence to support these roles was obtained by reheating a control gel (Fig. 5). Upon reheating, the G′ values steadily declined as the temperature rose from 25°C to approximately 77°C, with a rate of decline close to the increase in G′ seen during cooling. No additional changes in G′ values were observed between 77 and 90°C. Tan δ values exhibited similar behavior (Fig. 5). During the cooling phase from 90 to 78°C, tan δ values decreased. After that point, tan δ values increased slightly as the temperature dropped to 25°C. From this observation, it appears that the actual pattern of the gel matrix was established by molecular forces in the early cooling phase. During reheating, there was a gradual decrease in the tan δ value that reflected the increase observed during the latter stages of cooling. The sharp drop in tan δ values between 90 and 78°C was not reversible. Based on these thermoreversibility data, it appears that the addition of heat could have caused dissipation of hydrogen bonds, resulting in a reduction of the elastic modulus and a consequential increase in tan δ due to fewer crosslinks. The initial gel structure, however, remained intact, possibly stabilized by hydrophobic forces, electrostatic interactions and/or covalent bonds.

The frequency sweeps performed on the final gels containing GuanHCl showed significant differences in G′ values among the four treatments (Table 3). An increase in the molar concentration of GuanHCl yielded a decrease in the G′ modulus, reflecting fewer crosslinks within each gel system. These crosslinks are believed to be primarily hydrophobic and/or hydrogen-bonding in nature. Unlike the work with ovalbumin gels, where network integrity was maintained upon exposure to 8M urea (21), canola protein gels require noncovalent forces to develop, strengthen and/or maintain the structural network. If covalent bonds are present in the canola gels, their numbers are not sufficient to preserve the three-dimensional structure, or their position or distribution within the molecule is not conducive to gel formation.

The tan δ value obtained for the 3.0M GuanHCl sample at 1.0 Hz was significantly different from the tan δ values of the other three GuanHCl concentrations. No significant differences in tan δ values were found among the other treatments (Table 3). These results suggest that the actual crosslinking pattern is similar among the three samples that formed networks. This was reflected in the microstructure (not shown). In general, the networks formed in the presence of 0, 0.1 and 1.0M GuanHCl showed little variation in the actual arrangement of strands, although differences in the appearance of individual strands were quite noticeable. As the GuanHCl concentration was increased from 0.0 to 0.1M, thermal analysis data indicated increased stabilization of the protein. This may explain why the protein strands appeared to be more granular and compact than those of the control. When the GuanHCl concentration was increased to 1.0M, the protein network took on a swollen appearance. According to the Td and ΔH values obtained for this treatment, this

level of GuanHCl is capable of slight conformational changes. Disruption of both hydrophobic interactions and hydrogen bonds may have resulted in swelling of the strands and improved solubility of the macromolecule. Tan $\delta$ values indicated significant structural differences for the samples prepared in the presence of 3.0M GuanHCl. In fact, aliquots of the samples heated for microscopic analysis did not form gels.

*Effects of dithiothreitol (DTT).* Significant differences among thermal denaturation temperatures and enthalpies of denaturation confirmed the dependence of the molecule on covalent bonds for its native structure (Table 4). As a reducing agent, DTT is capable of disrupting existing disulfide crosslinks, causing destabilization and modification of a protein's native conformation (17). By increasing the DTT concentration from 0.0 to 0.15M, canola protein was progressively denatured.

The addition of DTT appears to have more effect on enthalpies of denaturation than on thermal denaturation temperatures. Given that disulfide linkages have high energy requirements for their disruption, it is possible the disruption of these bonds by DTT prior to thermal degradation greatly reduces the heat flow required to thermally denature the remaining structure. Keeping in mind that disulfide bonds are believed to be responsible for subunit association (22), it is possible the subunits themselves remain relatively intact upon exposure to DTT, thereby requiring temperatures of up to 70°C for their denaturation.

Protein dispersions to which DTT was added prior to heating demonstrated unique rheological characteristics throughout the course of gelation. In Figure 6A, the three DTT-treated samples exhibited structure development much earlier than the control. With reference to the thermal denaturation temperatures in Table 4, it is evident that gels begin to form after their respective temperatures of denaturation have been reached. Of particular interest is the fact that G′ values of the three DTT-treated samples are far greater than that of the control and that they appear to plateau by the end of the heating period. It seems that the cleavage of disulfide bonds prior to heating, along with the ensuing thermal degradation of the protein molecule, exposes binding sites that otherwise would have remained buried within the molecule. As a result, extensive crosslinking and subsequent structure formation are exhibited for these samples.

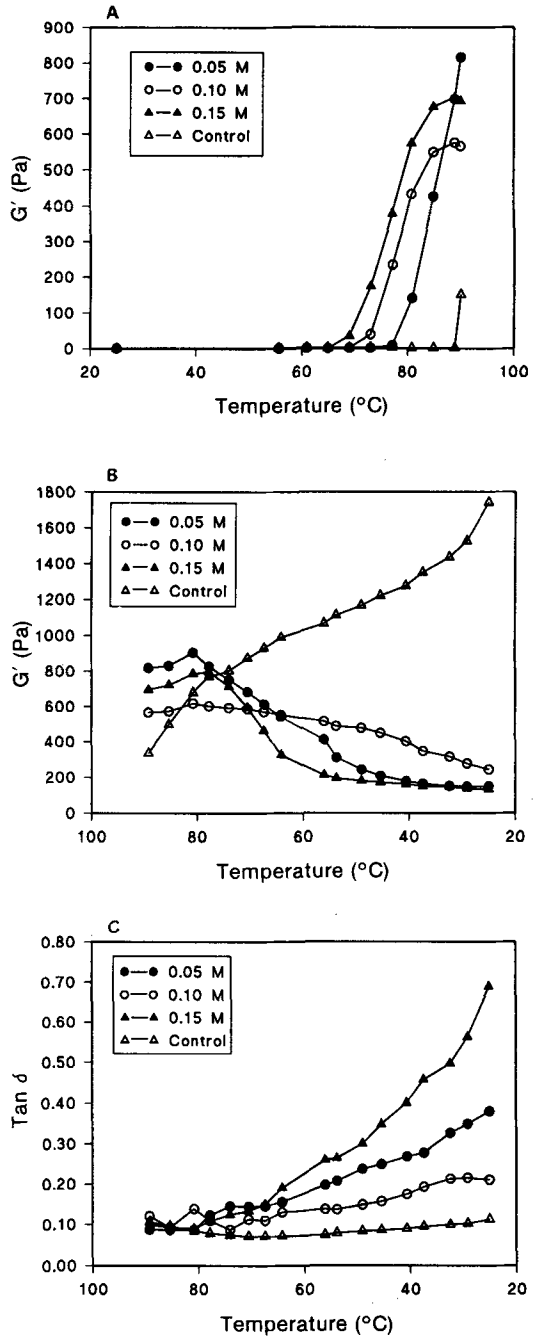


**FIG. 6. Effect of dithiothreitol concentration on rheological parameters as a function of temperature for 6% canola protein for: A, changes in G′ during heating; B, changes in G′ during cooling; and C, changes in tan $\delta$ during cooling.**

TABLE 4

**Effect of Dithiothreitol (DTT) Concentration on the Thermal Denaturation of 6% Canola Protein at pH 9 and Rheological Properties of 6% Canola Protein Gels (pH 9) at 1 Hz[a]**

| DTT concentration (M) | Td[b] (°C) | ΔH[c] (J/g protein) | G′[d] (Pa) | Tan $\delta$[e] |
|---|---|---|---|---|
| 0 | 81.01[f] | 18.44[f] | 1660[f] | 0.106[f] |
| 0.05 | 70.11[g] | 7.91[g] | 291[g] | 0.322[g] |
| 0.10 | 67.65[h] | 6.06[h] | 290[g] | 0.220[g] |
| 0.15 | 67.46[h] | 4.40[i] | 158[g] | 0.729[h] |

[a]Column values followed by the same letter are not significantly different ($P < 0.05$). Abbreviations as in Table 1.
[b]SE = 0.25.
[c]SE = 0.46.
[d]SE = 65.
[e]SE = 0.027.

The order in which the G′ values of the three DTT-treated samples increased did not correspond to the stepwise increase in DTT concentration. Perhaps there is an optimal level at which DTT causes the protein molecule to unfold in such a manner that the potential for crosslinking is maximized.

In Figure 6B, the G′ values of the three DTT-treated samples display an unusual downward trend during the cooling phase. After a slight increase at the onset, the 0.05 and 0.15M DTT samples exhibit a distinct drop in the G′

modulus as the temperature is reduced from 78 to 54°C. After this point, the values appear to stabilize. The decline and stabilization of G′ values for the 0.10M DTT sample was more gradual. From these results, it is evident that disulfide bonds play an important role in canola protein gelation. Through the addition of the disulfide modifying reagent prior to heating, it appears as if the intermolecular forces that dictate network formation are enhanced, whereas the forces responsible for initial stabilization of the structure are disrupted.

Tan δ values, as shown in Figure 6C, also display unique cooling patterns. Although all four samples possessed similar tan δ values at the onset, the tan δ values for the control remain relatively constant during the cooling phase, while the tan δ values for DTT-treated samples increase steadily. These results clearly demonstrate that DTT has a marked effect on the gelation mechanism. Between 90 and 67°C, similar tan δ values indicate that the actual arrangement of the gel structure is similar for all four samples. However, below 67°C, DTT-treated samples appear to lose their integrity. From these results, the forces responsible for stabilizing and/or strengthening the matrix in the mid-cooling range appear to have been disrupted. It is possible that disulfide crosslinks are responsible for this stabilization and/or strengthening. As a result, the structure of DTT-treated samples disintegrates, yielding "gels" with more fluid than elastic character.

Considering the strength of disulfide linkages, it was not surprising that gels are weakened by the reduction of these bonds. According to the G′ modulus, the control sample has significantly more crosslinks to support its three-dimensional network. With respect to tan δ values, significantly higher values for DTT-treated samples suggest that the structure of these gels was not as well established as that of the control, and that the gels had a strong viscous character.

The photomicrographs (not shown) illustrated the effect of DTT on the structure of canola gels. In comparison to the control, with its elongated chains and porous appearance, the structure of DTT-treated samples consisted of shorter, disconnected strands which were evenly distributed throughout the specimen. Given the delicate nature of the DTT samples, as reflected in the rheological parameters, it is possible that the networks were fragmented during specimen preparation, either by freezing or by slicing with the microtome. On the other hand, cleavage of disulfide bonds responsible for subunit association may have resulted in dissociation of the 12S canola protein into its smaller components. These subunits may not have been capable of forming extensive networks due to their size, shape and/or surface charge. This would affect the nature of the gels as evidenced by the reduction in the G′ modulus (Table 4). Lower G′ values provided proof of fewer crosslinks.

Likewise, high tan δ values (Table 4) indicate weaker structures for DTT-treated samples, and this was apparent in the photomicrographs. It is evident that the protein strands were not fully connected. This gives gels a fluid character because the liquid phase is not completely entrapped in a continuous three-dimensional network.

*Forces involved in network formation.* The main objective of this study was to determine the underlying forces responsible for canola protein gelation. By selectively altering the environment in which gelation took place, an attempt was made to identify the role of individual molecular forces. As no single environment can affect a specific molecular force, a composite of the data was used to gain an understanding of the gelation mechanism. Overall, it appears that hydrophobic interactions, along with a balance of attractive and repulsive forces, are responsible primarily for establishing canola gel networks; disulfide bonds, hydrogen bonds and electrostatic interactions contribute to gel stabilization and strengthening. These findings complement those of Gill and Tung (9) and Paulson and Tung (13) in terms of the types of interactions involved. However, the specific role of each of these forces has been addressed more closely in this study.

Of particular interest in this study was the strong evidence that supports the role of hydrophobic interactions in establishing the structure of canola gels. Hydrophobic interactions are favored at high temperatures. Rheological assessment revealed that structure development of most samples begins at the high temperatures encountered during the heating phase and/or the initial cooling period. Additional evidence that supports the role of hydrophobic forces in the establishment of canola gel networks was derived from the heating and cooling curves of environments that impact hydrophobic interactions. The addition of sodium salts to canola protein dispersions was aimed specifically at evaluating the contribution of hydrophobic forces to the gelation process. With as little as 0.1M salt, the salts exhibited a lyotropic effect and markedly influenced the extent to which gelation occurred. These results indicate that hydrophobic interactions definitely play a part in gelation, although their exact role could not be determined based on the salt data alone. The role of hydrophobic forces in the establishment of canola networks is supported by the GuanHCl and DTT effects. As disruption of hydrophobic interactions is a part of the denaturing mechanism of GuanHCl, the retardation of gel formation among GuanHCl-treated samples prior to heating may be related to the weakening of hydrophobic forces. Although DTT is a disulfide reducing agent, its addition to protein dispersions appears to enhance structure development during the heating phase. It is possible that the disruption of covalent bonds by DTT significantly alters protein conformation and thus promotes greater interactions among hyrophobic areas, resulting in greater structure formation among DTT-treated samples during heating. Although manipulation of pH was directed at evaluating its effect on electrostatic interactions, pH may affect hydrophobic forces as well. At high pH values, electrostatic repulsion can cause protein unfolding and exposure of previously buried reactive sites. In turn, this can lead to greater intermolecular interactions among hydrophobic groups. Enhanced gel formation at pH 8, 9 and 10 was accompanied by enhanced structure development at high temperatures. On the other hand, at low pH values, the greatest increase in G′ values occurred during the final phase of cooling. It was not possible to determine whether this lack of structure formation during the initial cooling was due to the unavailability of hydrophobic groups or to excessive electrostatic attraction, which favored aggregation reactions.

In addition to hydrophobic interactions, electrostatic interactions played a major role in establishing gel structure. At pH extremes, excessive repulsive charges are deemed responsible for the lack of gel formation.

Electrostatic interactions are believed to also be important in the overall balance of attractive and repulsive forces contributing to strong network formation. Disruption of this balance, either through direct interactions or conformational changes, may account for the inhibition of network formation with GuanHCl and the promotion of initial network formation by DTT.

Once the three-dimensional network of the gel was established, there was a solidification and/or stabilization of the structure. This is reflected by a change in the slope of the cooling curve between 78 and 55°C. Disulfide and hydrogen bonds are thought to be the principal molecular forces behind this gel stabilization. The unique cooling curves were obtained by heating canola protein pretreated with DTT, and there was not sufficient evidence to determine the involvement of disulfide bonds in the gelation process. Support for the role of disulfide bonds as a stabilizing force was derived from the thermal reversibility curve where previously established forces retained gel structure at temperatures above 78°C. Failure of a gel to melt upon heating is usually indicative of the presence of covalent bonds. In this case, disulfide bonds were essential to the solidification of the gel structure.

The role of hydrogen bonding in gel strengthening is believed to be particularly important during the final cooling phase. Given that hydrogen bonding is favored at low temperatures, the gradual increase in G′ values during the latter stage of gelation is attributed to this molecular force. This is supported by the reduction in the rate at which G′ increased during the latter stages of cooling in the presence of GuanHCl. Additional evidence for the contribution of hydrogen bonds to gel strengthening is provided by the thermal reversibility curves where G′ values decreases between 25 and 78°C during reheating. This indicates a loss of gel strength and a reduction in the number of crosslinks due to dissipation of hydrogen bonds with increasing temperature.

## ACKNOWLEDGMENTS

The assistance of the Natural Sciences and Engineering Research Council of Canada in supporting this research is gratefully acknowledged. E.D. Murray and M.A.H. Ismond provided valuable advice during the initial stages of this project.

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[Received January 4, 1993; accepted July 5, 1993]