Heat-Induced Gel Formation by Soy Proteins at Neutral pH

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Heat-induced gel formation by soy protein isolate at pH 7 is discussed. Different heating and cooling rates, heating times, and heating temperatures were used to elucidate the various processes that occur and to study the relative role of covalent and noncovalent protein interactions therein. Gel formation was followed by dynamic rheological measurements. Heat denaturation was a prerequisite for gel formation. The gelation temperature (84 °C) was just above the onset denaturation temperature of glycinin. The stiffness of the gels, measured as the elastic modulus, G', increased with the proportion of denatured protein. An increase in G' was also observed during prolonged heating at 90 °C. This increase is explained by the occurrence of rearrangements in the network structure and probably also by further incorporation of protein in the network. The increase in G' upon cooling was thermoreversible indicating that disulfide bond formation and rearrangements do not occur upon cooling.

INTRODUCTION

Soy proteins are often used in food products to improve texture. In this respect the most important property is their ability to form a gel with a good water-holding capacity upon heating. It is expected that in the future soy proteins will play a major role as a meat replacement because of their high nutritional value. However, despite their importance and all the research performed, gel formation by soy proteins is still not clearly understood.

Soy protein isolate consists of two major components, β-conglycinin and glycinin, which are also called 7S and 11S globulin. β-Conglycinin is less heat-stable than glycinin; the onset denaturation temperature of β-conglycinin is about 70 °C, and that of glycinin is about 80 °C (at neutral pH and no salt added) (*1*). Denaturation is believed to be a prerequisite for gel formation (*2*), so purified β-conglycinin will form a gel at lower temperatures than purified glycinin, which is indeed observed by Nagano and co-workers (*3*).

Heat-induced gel formation by soy proteins and the molecular interactions involved were studied by various researchers (*4−9*). Hereto, protein structure destabilizers and stabilizers, such as urea, SDS, β-mercaptoethanol, NaSCN, and NaCl, were used. For soy protein isolate and purified glycinin, it was concluded that disulfide bridges are involved in the gelation process, whereas in β-conglycinin gels they do not play a role. For all three protein systems (glycinin, β-conglycinin, and soy protein isolate), noncovalent interactions such as hydrogen bonding and hydrophobic interactions play a role in gelation. It is not yet clear in which stages of gel formation these covalent and noncovalent bonds are important.

In this article, we used a somewhat different approach to study heat-induced gel formation. To elucidate the different processes during gel formation, heating conditions were varied. Dynamic rheological measurements were used to follow gel formation by soy protein isolate at pH 7 and to study whether the protein−protein interactions were covalent or noncovalent.

MATERIALS AND METHODS

Preparation of Soy Protein Isolate. Soy protein isolate (SPI) was prepared from mildly treated, defatted PDI80 soy flakes (Cargill BV, Amsterdam, The Netherlands). The flakes were milled in a Fritsch Pulverizette 14702 using a 0.5-mm sieve. Milling was performed in the presence of solid CO_2 (volume ratio flakes:CO_2 is 4:1) to prevent heat denaturation of the proteins. The flour was suspended in a 100 mM Tris−HCl buffer of pH 8 in a 1:10 ratio (w/v), and stirred at room temperature for 1 h. After removal of the insoluble parts by centrifugation (30 min, 12,000*g*, 10 °C), the supernatant was brought to pH 4.8 with 2 M HCl to induce precipitation of the soy proteins. After 2 h at 4 °C the dispersion was centrifuged (30 min, 12,000*g*, 10 °C). The precipitate was washed twice with a 10 mM sodium acetate buffer of pH 4.8 in a 1:8 ratio (w/v) and freeze-dried afterward. This material will be referred to as SPI. The protein content was 97% using N × 6.25.

Preparation of Protein Dispersions. Protein dispersions were prepared by suspending SPI in double-distilled water in higher concentrations than required for the experiments. After the suspension was stirred for 1 h at 4 °C, it was brought to pH 7 with 0.5 M NaOH, after which the volume of the dispersion was adjusted by adding double-distilled water to obtain the desired protein concentration. The protein dispersions were stirred overnight at 4 °C to get the protein better dissolved. The preparation of the dispersions was performed at 4 °C to prevent proteolysis by endogenous enzymes.

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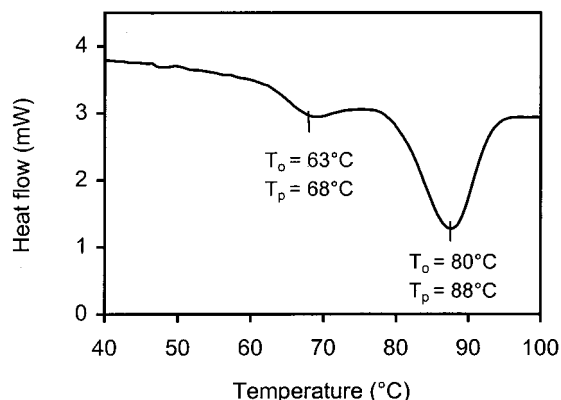
Figure 1. DSC-thermogram of a 10% soy protein isolate dispersions in double-distilled water (pH 7). The onset (T_o) and peak (T_p) denaturation temperatures of both endotherms are given.

Figure 2. Degree of denaturation (■) and storage modulus G' after heating for 1 h at maximum temperature (◆) and after cooling (▲), of 10% soy protein isolate dispersions in double-distilled water (pH 7) as a function of heating temperature.

Small Deformation Experiments. Gel formation was followed by dynamic measurements using a Bohlin CVO rheometer with a serrated concentric cylinder geometry (C25). The measurements were performed at a constant strain of 0.01, which was within the linear region, and at an angular frequency of 0.63 rad/s. To prevent solvent evaporation, a thin layer of soy oil was put on top of the samples. To induce gel formation, protein dispersions were consecutively heated from 20 to 90 °C at a heating rate of 1 K/min, kept for 1 h at 90 °C, and cooled to 20 °C at a cooling rate of 1 K/min, unless stated otherwise. Applied variations in the above-mentioned temperature profile were heating/cooling rate (1 and 5 K/min), heating time at 90 °C (0, 1, and 5 h), and heating temperature (80−95 °C). After gel formation, frequency dependence was studied from 0.0063 to 63 rad/s at several temperatures.

Differential Scanning Calorimetry (DSC). The degree of protein denaturation in soy protein isolate as a function of heating temperature was determined by differential scanning calorimetry and calculated by dividing the change in enthalpy, ΔH, associated to the denaturation peak of glycinin of a preheated suspension by that of an unheated suspension. Thereto, protein dispersions were first heated in 0.9-mL stainless steel vessels in a micro-DSC (Setaram, France) from 20 °C to a temperature ranging from 76 to 94 °C at a heating rate of 1 K/min, maintained at that temperature for 1 h, and cooled to 20 °C at a cooling rate of 1 K/min. Consecutively, the sample was scanned from 20 to 115 °C at a scanning rate of 1 K/min. A sample that was not preheated was scanned in the same way.

The temperature at which denaturation starts, the onset denaturation temperature T_o, was calculated by taking the intercept of the baseline and the extrapolated slope of the peak. For the peak denaturation temperature T_p, the temperature at maximum heat flow was taken.

RESULTS

DSC. A DSC-thermogram of a 10% SPI dispersion that was not preheated is shown in **Figure 1**. Two endothermic transitions were observed caused by heat denaturation of β-conglycinin at the lowest temperature and of glycinin at the highest temperature (*1*). The onset (T_o) and peak (T_p) denaturation temperatures were 63 and 68 °C for β-conglycinin and 80 and 88 °C for glycinin. Heating the dispersion for a second time showed a DSC-thermogram without endotherms indicating that heat-induced denaturation of the proteins was followed by irreversible processes such as aggregation.

Figure 2 shows the degree of denaturation of SPI dispersions that were heated for 1 h at temperatures ranging from 76 to 94 °C. At all these heating temperatures the second run DSC curve did not show an endothermic transition of β-conglycinin, which means that β-conglycinin was already denatured in the first run. As the heating temperature increased, the degree of denaturation of the glycinin fraction increased. At 88 °C, SPI was completely denatured.
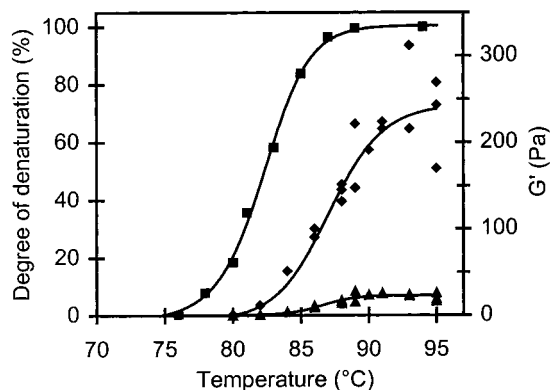
Gel Formation. In **Figure 3A** a typical example of a gelation curve of soy protein isolate at pH 7 as a function of time is presented. At 84 °C ($t = 64$ min), the storage modulus G', which is a measure of the stiffness of the gel, started to increase; this temperature is defined as the gelation temperature. G' kept increasing upon further heating, but a much stronger increase in G' was observed upon cooling. In **Figure 3B** the same gelation curve is presented (solid line), but in this case as a function of temperature. A second curve (dotted line) is plotted which resembles reheating of the gel from 20 to 90 °C at 1 K/min. The second heating curve overlapped the cooling curve for the largest part indicating that gel stiffening during cooling was almost completely thermoreversible.

The effect of heating time at 90 °C on the gelation curves is shown in **Figure 4**. G' increased gradually on prolonged heating at 90 °C, resulting in higher G' values just after the heating step, and even higher values after cooling. The effect of heating and cooling rate was studied for 1 and 5 K/min (**Figure 5**). The actual maximum cooling rate was in the latter case 3 K/min. At a heating rate of 1 K/min gelation started at a lower temperature than at 5 K/min, which resulted in a stiffer gel after cooling. Cooling rates did not seem to affect G' during and after cooling, when heating was performed at a heating rate of 1 K/min (data not shown).

As for the degree of denaturation, the effect of heating temperature on gel formation was studied. **Figure 2** shows G', immediately after heating for 1 h, and G', after cooling to 20 °C, as a function of heating temperature. Gels were formed at heating temperatures higher than 80 °C. Up to 90 °C, the stiffness of the gels increased with increasing heating temperatures. At temperatures higher than 90 °C, G' seems to reach a plateau value. It is not clear whether a plateau value or a maximum in the curve is obtained, because from this point the scatter in the data became large.

Combination of the *y*-data from **Figure 2** gives G' as a function of the degree of denaturation (**Figure 6**). At least 20% denaturation of glycinin was required to achieve notable gel formation. As more protein became denatured, higher G' values were observed.

Frequency Dependence. Figure 7 shows the frequency dependence of the storage modulus, G', and the loss tangent, *tan* δ, at 20, 50, and 90 °C. At 20 °C, log G' increased linearly with increasing log frequency and *tan* δ was only slightly dependent on frequency. At temperatures of 50 °C and higher, G' and *tan* δ became more frequency dependent (**Figures 7** and **8**).

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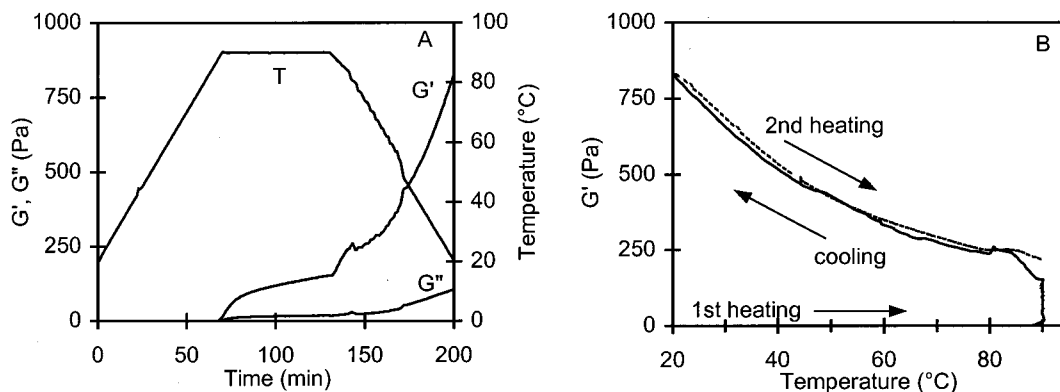
Figure 3. Dynamic moduli, G' and G'', of an 11% soy protein isolate dispersion in double-distilled water at pH 7 as a function of time during a heating and cooling cycle (T) (A) and the storage modulus G' as a function of temperature (B). In B, the full line represents the first heating and cooling curve; the dotted line represents the second heating curve.
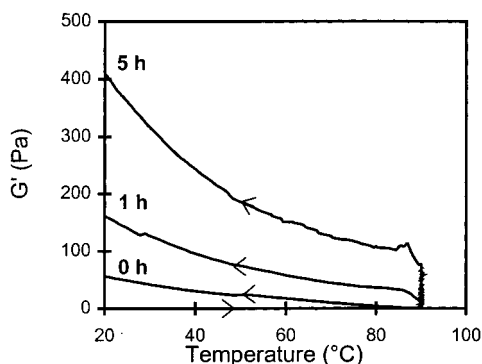
Figure 4. Storage modulus G' during heating and subsequent cooling of 10% soy protein isolate dispersions in water (pH 7) for heating times at 90 °C of 0, 1, and 5 hours, respectively. Heating and cooling rates were 1 K/min. >, heating; <, cooling.
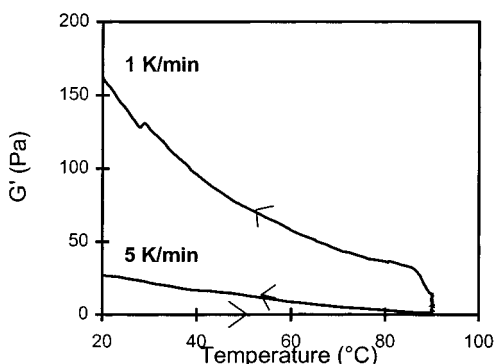

Figure 5. Storage modulus G' during heating and subsequent cooling of 10% soy protein isolate dispersions in water (pH 7) as a function of heating rate (indicated). Heating time at 90 °C was 1 h.

DISCUSSION

Below, we will discuss consecutively the different stages in the gelation process: the onset of gel formation and the role of protein denaturation therein, the development of the storage modulus G' and the occurrence of rearrangements in the network structure during (prolonged) heating, and the reversible stiffening of the gel on cooling.

The temperature at which G' started to increase (84 °C), the onset of gel formation, is between the onset and peak denaturation temperature of glycinin (**Figure 1**). At a heating rate of 5 K/min, G' started to increase later than at 1 K/min, i.e., 3 min after the program had reached 90 °C. At higher heating rates denaturation temperatures are higher (e.g. (*10*)), which would
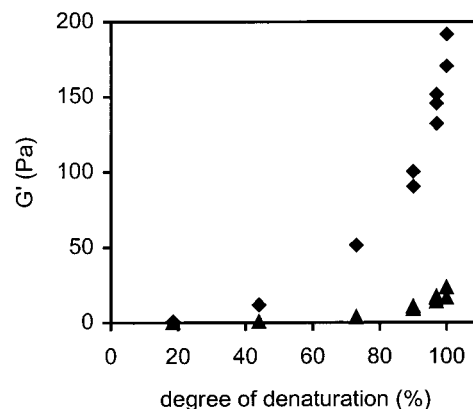

Figure 6. Storage modulus G' after heating for 1 h at maximum temperature (◆) and after cooling (▲) of 10% soy protein isolate dispersions in water (pH 7) as a function of the degree of protein denaturation.
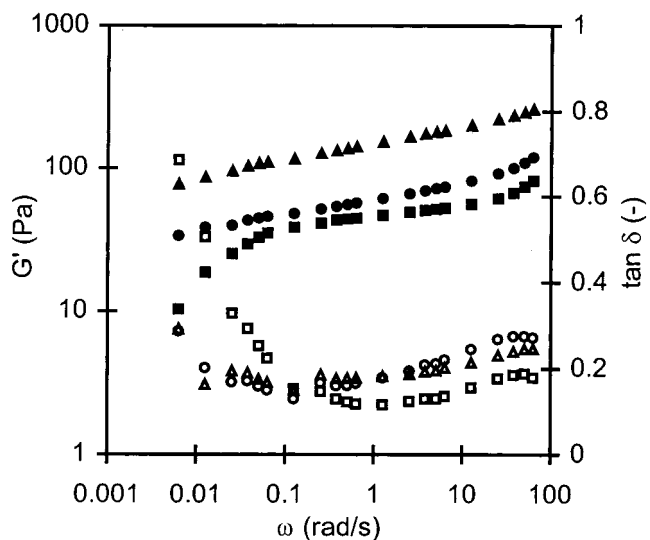
Figure 7. Storage modulus G' (closed symbols) and loss tangent *tan δ* (open symbols) of a 10% soy protein isolate gel (pH 7) as a function of angular frequency, ω, at 20 (▲), 50 (●), and 90 °C (■). Gel was prepared according to the standard procedure.

explain the higher gelation temperature at 5 K/min compared to 1 K/min. Moreover, aggregation and network formation may take some time. The results confirm the idea that heat denaturation is a prerequisite for gel formation (*2*). It is remarkable that gelation did not start at lower temperatures,
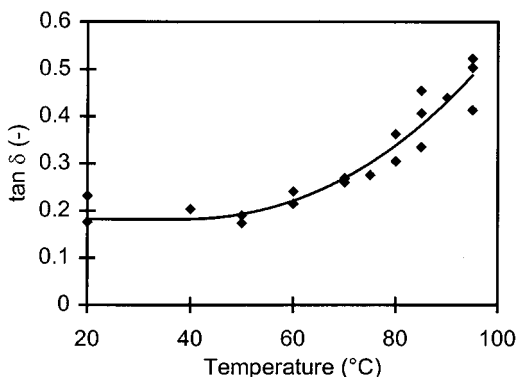
Figure 8. Loss tangent, *tan δ*, of a 10% soy protein isolate gel (pH 7) as a function of temperature at angular frequency, ω = 0.0063 rad/s. Gel was prepared according to the standard procedure.

namely after heat denaturation of β-conglycinin, which has a peak denaturation temperature of 68 °C at these conditions. Likely, the explanation is that the β-conglycinin concentration was too low (<4 wt % of the total dispersion) to result in a notable increase of G' (*11*).

The conclusion that protein denaturation is a prerequisite for gel formation is confirmed by the data in **Figure 6**. This figure shows that a certain amount of protein had to denature before a gel was formed and that the storage modulus increased with the amount of denatured protein.

In principle, heat denaturation of proteins is a reversible process. At each temperature, a specific equilibrium exists between proteins in the native state and proteins in the denatured state. However, on (partial) unfolding of soy proteins, functional groups such as sulfhydryl groups and hydrophobic groups become exposed and immediately interact with each other leading to irreversible protein aggregation and network formation (gelation). The equilibrium of native and denatured protein is restored continuously after aggregation of the denatured protein, until the point where no native protein is left. In practice, this means that heat denaturation of proteins is an irreversible process.

The data show (a) broad endothermic transitions in the DSC thermogram (**Figure 1**) and (b) that different amounts of denatured glycinin could be obtained by using a range of heating temperatures (**Figure 2**). This is explained by the fact that both the β-conglycinin and the glycinin fraction are composed of several genetic variants, which have different thermal stabilities (*12−14*). For the data in **Figure 2** this means that at a certain degree of denaturation some of the glycinin variants are denatured and some are still native. An additional reason for obtaining different amounts of denatured protein is that the heating conditions might have been such that denaturation was not yet completely irreversible.

After the onset of gelation, an increase in G' is observed on further heating. G' increased because more protein becomes incorporated into the network leading to a further built-up of the network structure. Apart from that, G' might have increased by rearrangements in the network. A type of rearrangement that might occur is fusion of the protein aggregates in the strands, which results in an increase of protein−protein interactions per cross-section, and so results in denser and stiffer strands. Another type of rearrangement involves fracture or yielding of strands leading to regions with high and low densities of protein (*15−18*). This fracture is likely to happen in thin strands and/ or when the average lifetime of the protein−protein bonds is short. Breaking of the strands due to relaxation of the intermolecular protein bonds is induced by thermal motion. There are

no indications that the increase in G' during prolonged heating is caused by evaporation.

An indication for the occurrence of rearrangements in the network structure during heating was obtained from the frequency dependence data (**Figures 7** and **8**). High *tan δ* values (= G''/G') at low frequencies (ω) show that gels have a more viscous behavior at longer time scales ($t = 1/\omega$) and that the average lifetime of the protein−protein bonds is rather short. Because such short-lived bonds are more likely to break and reform due to thermal motion, it indicates that rearrangements can occur (*15, 19*). The high *tan δ* at 90 °C (**Figures 7** and **8**) shows that rearrangements in soy protein gels possibly occur during prolonged heating at high temperatures in contrast to temperatures lower than 50 °C.

Another indication that rearrangements take place at high temperatures was obtained by confocal scanning laser microscopy (CSLM). Gels that were heated for 4 h at 95 °C showed somewhat larger aggregates and significantly more contrast between protein and background than gels that were heated for 1 h (data not shown). It is likely that rearrangements induced the changes on prolonged heating as observed in the micrographs and that these changes affected the stiffness of the gels.

No distinction could be made between the contribution to G' by continuous protein incorporation into the network and that by rearrangements. It is obvious that the steep increase of G' at the onset of gelation is caused by further incorporation of protein after the initial network is formed. Yet, we do not know if the slow increase in G' on prolonged heating at 90 °C (**Figure 3**) is primarily caused by additional protein built into the network or by rearrangements. The former is the case for whey protein gels (*20*) while the latter occurs in casein gels (*18*). For soy proteins, no literature on the occurrence of these two processes or their importance for stiffening of the gels was found.

It is not clear why gels prepared at a heating rate of 5 K/min had a lower G' than gels at heated at 1 K/min. The same phenomenon has been observed for other globular proteins such as β-lactoglobulin (*21*), vicilin, and ovalbumin (*22*). The higher heating rate might have affected the aggregation kinetics resulting in gels with a different network structure (different pore size, strand thickness, or curvature). It is known that differences in network structure affect G' (*23−25*). Gels with curved strands have lower G' values than gels with predominantly straight strands, because bending deformation involves less energy than stretching deformation, and thin strands are easier to bend than thick strands (lower G').

It is known that formation of intermolecular disulfide bonds via thiol-disulfide interchange and oxidation reactions plays a role in aggregation of soy protein (*26*). We doubt, however, the importance of disulfide bridges for the stiffening of the initially formed network during heating. Disulfide bridges, being covalent bonds, have a high bond strength compared to noncovalent bonds (*27*) and will therefore not be broken over time scales of minutes, which is in contrast with the results obtained for G' and *tan δ* at low ω and 90 °C (time scale = $1/\omega$) (**Figure 8**). This implies that soy protein isolate gels do not consist of a so-called covalent network and agrees with earlier conclusions that soy protein gels primarily consist of noncovalent bonds (*5, 28*). The exact nature of the noncovalent protein−protein bonds (hydrogen bonds, hydrophobic or electrostatic interactions, etc.) that exist in the network was not studied by us.

The last stage of the gelation curve, the gel stiffening during cooling, was almost completely thermoreversible (**Figure 3B**), which agreed with findings by Catsimpoolas and Meyer (*28*)

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and van Kleef (*5*), and independent of cooling rate. The reversibility is an indication that no covalent bonds are formed during this stage and that no rearrangements take place involving fracture of strands, because these processes are irreversible. Also, the absence of an effect of cooling rate implies that no chemical reactions take place between protein molecules, because the reaction kinetics would be affected by the cooling rate. The increase in G' upon cooling is probably caused by a decreasing mobility of the proteins with decreasing temperature, which allows enhanced bond formation in and between the protein molecules.

The bump in the cooling part of the gelation curve (**Figure 3A**: $t = 130-150$ min; **Figure 3B**: $T = 80-90$ °C) is partly explained by malfunctioning of the cooling program; G' reacted strongly on temperature fluctuations. However, rearrangements in the network structure, resulting in an increase of G', might also partly cause the bump in the cooling stage, because at 85 °C these may still take place as indicated by the high *tan δ* at low ω (**Figure 8**). The occurrence of rearrangements also explains why G' was not fully thermoreversible at temperatures above 85 °C.

Summarizing, heat-induced gel formation by soy proteins involves several processes such as denaturation, aggregation (in which disulfide bridges play a role), network formation, and gel stiffening. Gel stiffening during prolonged heating is caused by rearrangements in the network structure and probably to some extent by further incorporation of protein into the network. Gel stiffening during cooling is a thermoreversible process and does, therefore, not involve disulfide bond formation or rearrangements in the network structure. The bonds formed during gel formation are mainly of noncovalent nature.

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