Gelation Phenomena of Soybean Globulins. I. Protein-Protein Interactions

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ABSTRACT

Heating of aqueous soybean globulin dispersions (concentration > 8%) activates the protein sol to the progel state which is characterized by a marked increase in apparent viscosity measured at the ambient temperature of the progel. The gel is obtained by cooling the progel, and an additional increase in apparent viscosity is observed. The transition is reversible, and the gel can be converted to the progel state by heat and then cooled again to obtain the gel. The progel and the gel are converted by excess heat or the action of chemical reagents to a "metasol" which does not form a gel on cooling. The effects of pH, temperature, and ionic strength on the formation of both the progel and the gel are very pronounced. Both the strength and maximum temperature of formation of the progel and gel are affected by pH. The bonds involved in the sol-progel and progel-gel transitions appear to be primarily of noncovalent nature.

The importance of the rheological properties of edible soybean proteins with respect to their use in food systems has been adequately discussed by Circle et al. (1). Previous studies (1) have shown that the gelation of aqueous dispersions of soybean globulins is dependent on temperature, pH, time of heating, and protein concentration. The present studies are concerned with a further investigation of the effect of these factors on the sol-progel-gel transitions in an attempt to characterize the mechanism of gelation and some of the molecular forces involved.

Soybean globulins represent a heterogeneous mixture of components, as has been shown by ultracentrifugal (2,3), electrophoretical (4,5,6,7), and immunochemical (8,9) analyses. In addition, association-dissociation phenomena observed under different experimental conditions (10,11,12,13,14) contribute to their complexity. Although gelation studies on isolated soybean globulins (6,12,15,16,17,18) are highly desirable from a theoretical viewpoint, the pure globulins are difficult to obtain in quantities large enough for macroscopic viscosity measurements. Furthermore, such studies may not be characteristic of the properties of the protein mixture which is commercially available for use in food applications. Therefore, for practical reasons, the present data were obtained with a mixture of soybean globulins separated from the whey proteins and other contaminants by isoelectric precipitation.

MATERIALS AND METHODS

Soybean Globulins

Soybean globulins were obtained by aqueous extraction of defatted soybean flakes at pH 8 (ratio of flakes to water 1:20), clarification of the extract by centrifugation, and precipitation of the globulins by acidification to pH 4.5 with HCl. The protein suspension was then washed with water, adjusted to pH 7 with NaOH, and spray-dried.

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Dispersion and Gelation

Aqueous dispersions of the protein were prepared on a weight-percentage basis, and placed in a Sorvall Omni-Mixer operated at full speed for 3 min. to ensure complete dispersion. Air in the dispersions was removed by centrifugation for 1 min. at 250 X g. All gelation experiments were carried out by placing the protein samples in 25 by 150-mm. test tubes which were stoppered and heated to the specified internal temperatures in a water bath. Internal temperatures were recorded with a YS1 telethermometer equipped with a glass thermistor probe. After a suitable time interval of heating, the tubes were immediately removed and apparent viscosities were measured either immediately or after cooling at 4°C. for 1 hr.

Viscosity Measurements

A Brookfield LVT viscometer was used to determine viscosities in fluid and gelled dispersions in conjunction with the Brookfield Helipath which permits the measurement of apparent viscosity of undisturbed structure by slowly lowering a T-shaped spindle at various relatively slow rotational speeds through the dispersion. The calibration of this instrument was checked with two different viscosity standards. Data are reported as "apparent viscosity" in view of the thixotropic properties of the soybean globulin gels.

Melting Points

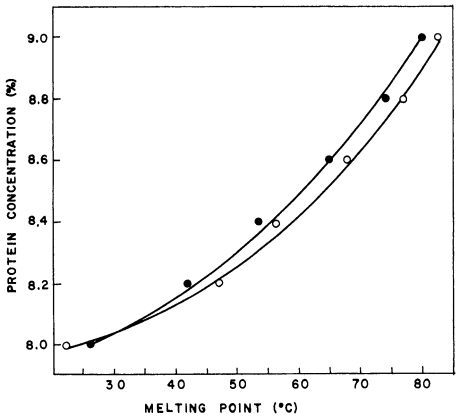
Melting points of the gels were determined by a modification of the method described by Bello et al. (19) for gelatin gels. Soy globulin dispersions were placed in 15 by 120-mm. test tubes which were stoppered and then heated at 80°C. for 30 min. The progels were then cooled at 4°C. for 1 hr. Steel balls (3.525 ± 0.015 g.; 1.0-cm. diameter) were placed under the surface of the gels and the tubes were heated at the rate of 2.5°C. per min. The temperature at which the ball reached the bottom of the tube was taken as the melting point of the gel (± 0.5°C. reproducibility of melting points).

RESULTS

Melting Points of Gels

The minimum concentration to obtain a self-supporting gel network with a heated aqueous dispersion of soybean globulins at pH 7 and room temperature is approximately 8%. The gels were prepared by heating the protein dispersion at 80°C. for 30 min., then cooled for 1 hr. at 4°C. The heating process activates the protein to the progel state which is characterized by a significantly higher viscosity than the unheated material. Cooling of the progel results in "setting" of the gel. The sol-progel transition is irreversible, for reasons that will be discussed later. However, the progel-gel transition is reversible under experimental conditions described here.

Heating of a gel results in "melting" to the progel state. The melting point is a function of the protein concentration, as shown in Fig. 1. Gels aged for 24 hr. at 4°C. showed little difference in melting point in comparison with those aged for 1 hr. (Fig. 1). A plot of log c vs. 1/Tg (where c is the protein concentration in g. per



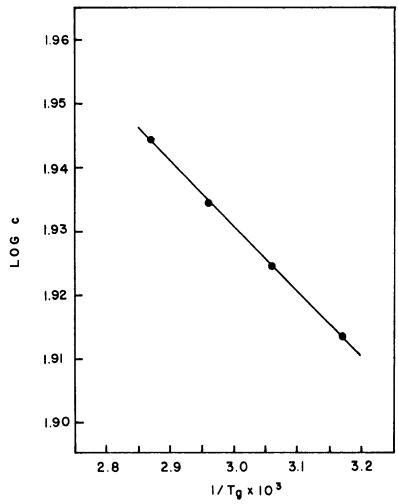


Fig. 1 (left). Melting points of soybean globulin gels as a function of protein concentration: Solid circles, gels aged for 1 hr. at 4°C.; open circles, gels aged for 24 hr. at 4°C.

Fig. 2 (right). Relation between protein concentration (c, g. per liter) and absolute temperature of gel melting.

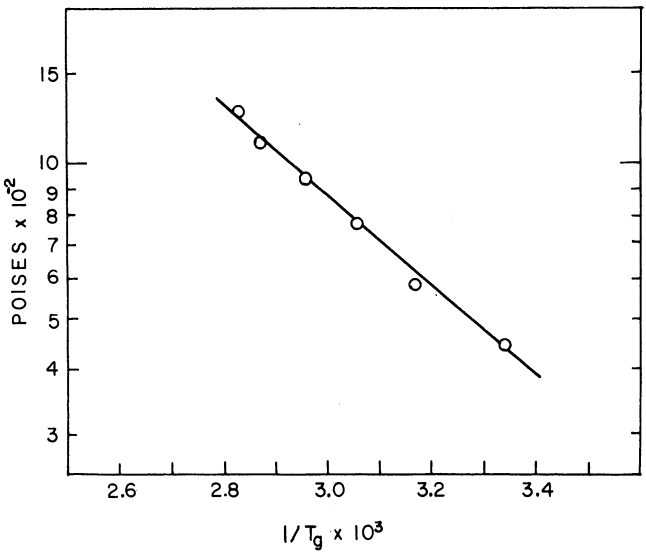


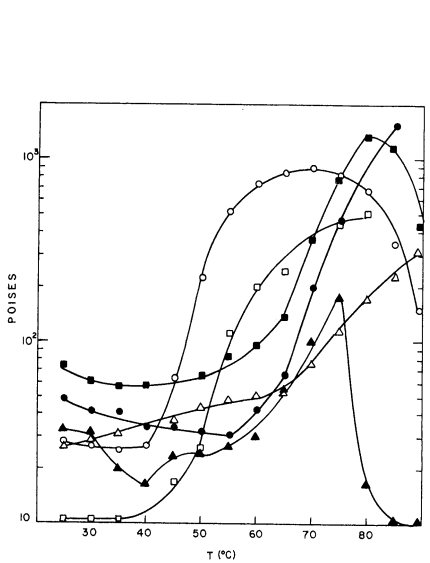
Fig. 3. Relation between apparent gel viscosity and absolute temperature of gel melting.

liter and Tg is the absolute temperature of melting obtained from data shown in Fig. 1) yields a straight line (Fig. 2).

Within the limits of concentration indicated in Fig. 1, the logarithm of the apparent viscosity of the gel plotted against the reciprocal of the absolute temperature of the melting point also produces a straight line (Fig. 3). This indicates that the viscosity of the gel is directly related to the protein concentration, since both parameters have the same relation to the absolute temperature of melting.

Effects of pH and Temperature on the Viscosities of the Progel and Gel

Aqueous soybean globulin dispersions (10% concentration) adjusted to the desired pH were heated at the rate of 0.5°C. per min. At each experimental temperature (internal temperature of the progel) a tube was removed from the water bath and the viscosity was determined immediately; at the same time a duplicate dispersion, heated to the same temperature, was removed and cooled at 4°C. for 1 hr. The viscosity of the "hot" dispersion (at ambient temperature) was taken as that of the progel state and the viscosity of the "cooled" dispersion (measured at 25°C.) as representative of the gel state. Figures 4 and 5 show the changes in viscosity of the progel and gel as function of temperature and pH. At any pH value, the viscosities of both the progel and gel, after usually an initial decline, rise with increase in temperature until a maximum is reached, which may



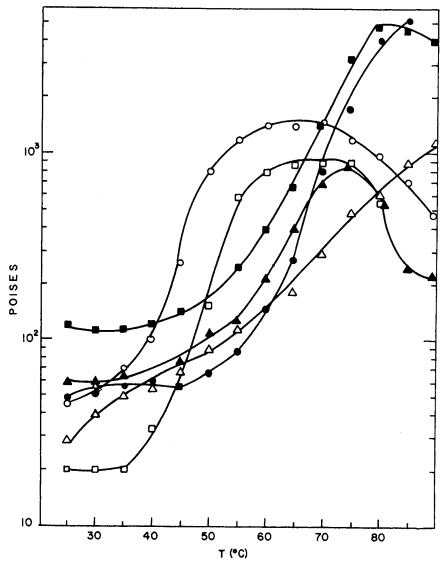


Fig. 4 (left). Effects of pH and temperature on the apparent viscosity of the progel: 10% soybean globulins (w./v.): open circles, pH 1.0; open squares, pH 2.0; open triangles, pH 6.0; solid circles, pH 7.0; solid squares, pH 8.0; solid triangles, pH 10.0.

Fig. 5 (right). Effects of pH and temperature on the apparent viscosity of the gel: 10% soybean globulins (w./v.):open circles, pH 1.0; open squares, pH 2.0; open triangles, pH 6.0; solid circles, pH 7.0; solid squares, pH 9.0; solid triangles, pH 10.0.

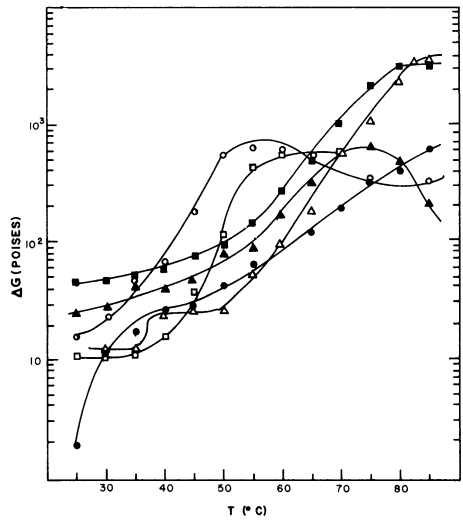


Fig. 6. Effects of pH and temperature on the apparent viscosity (ΔG) gained during cooling of the gels from the progel state: 10% soybean globulins (w./v.): open circles, pH 1.0; open squares, pH 2.0; solid circles, pH 6.0; open triangles, pH 7.0; solid squares, pH 9.0; and solid triangles, pH 10.0.

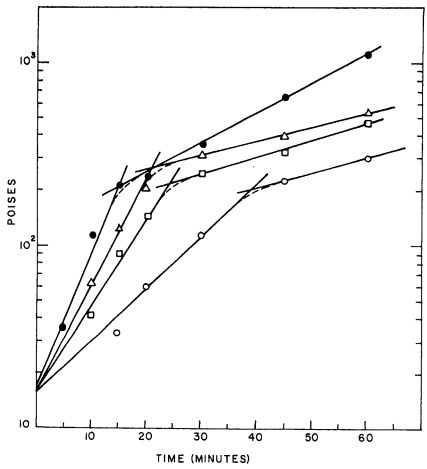
be followed by a drop (with formation of a metasol) as the temperature is raised beyond this point. The effect of excess heating in causing a drop in viscosity is more pronounced at very acid or very alkaline pH. Highest viscosity values are obtained at neutral or mildly alkaline pH. Either an increase or a decrease in pH produces weaker gels. Figure 6 shows the viscosity (in poises) gained during cooling of the gels. The calculated viscosity points (ΔG) were obtained by subtracting the progel viscosity from the gel viscosity.

Gelation as a Function of Time and Temperature at pH 7

Samples of an aqueous soybean globulin dispersion (10% concentration, pH 7) were heated for various time intervals at 65°, 70°, 75°, and 80°C. Separate tubes containing aliquots of the dispersion were used for each viscosity measurement in both the progel and gel states. The viscosities of the progel as a function of time and temperature are shown in Fig. 7 and those of the gel in Fig. 8. It is seen that the viscosity of the progel increases rapidly at first with time until a value of about 200 poises is reached. This appears to be the minimum average viscosity value for the appearance of a self-supporting network. Above this value (which may be a transition point) the viscosity of the progel continues to increase with time but less rapidly. The apparent viscosities of the gels, heated at various temperatures and for various times, fit curves with no apparent transition point, since most of the observed viscosities had values above 200 poises.

Thermal Conversion of Gel to Progel

Gels were formed by heating 10% aqueous dispersions of soybean globulins of the desired pH (at 80°C. for pH 7.0, and at 75°C. for pH 9.5 and pH 2.0) for 30



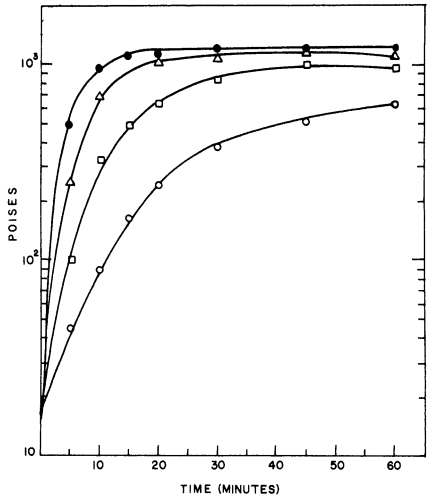


Fig. 7 (left). Apparent viscosity of the progel as a function of time and temperature: 10% soybean globulins (w./v.), pH 7: open circles, 65°C.; open squares, 70°C.; open triangles, 75°C.; solid circles, 80°C.

Fig. 8 (right). Apparent viscosity of the gel as a function of time and temperature: 10% soybean globulins (w./v.), pH 7: open circles, 65°C.; open squares, 70°C.; open triangles, 75°C.; solid circles, 80°C.

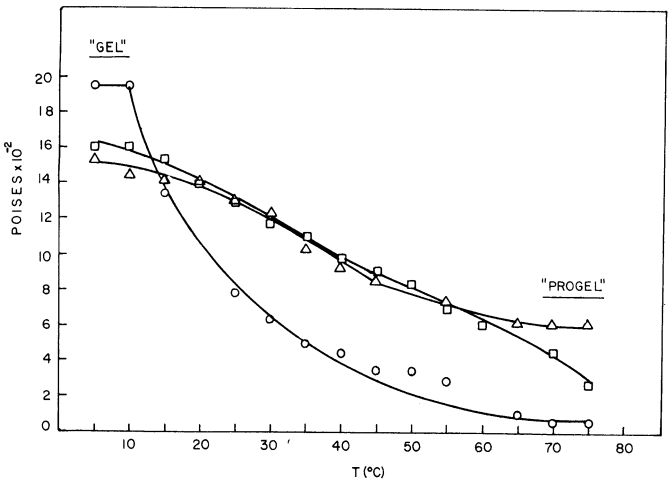
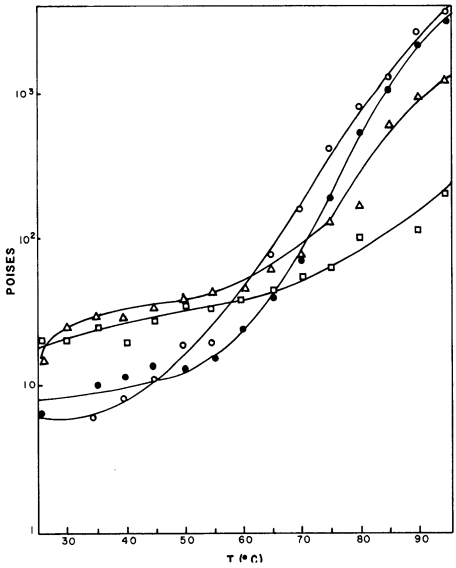


Fig. 9. Gel-to-progel conversion curves as a function of pH and temperatures; 10% soybean globulins (w./v.), gels formed by heating at 80° (pH 2.0) and at 75°C. (pH 7.0 and pH 9.5) for 30 min. and cooled at 4°C. for 1 hr.: open squares, pH 2.0; open triangles, pH 7.0; open circles, pH 9.5.



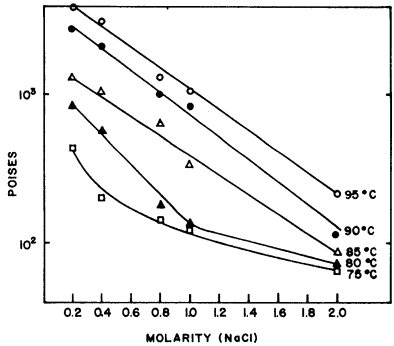


Fig. 10 (left). Apparent viscosity of 10% soybean globulin gels (w./v.), pH 7.0, containing different concentrations of NaCl: open circles, 0.2M; solid circles, 0.4M; open triangles, 0.5M; and open squares, 2.0M.

Fig. 11 (right). Apparent viscosity of 10% soybean globulin gels (w./v.), pH 7.0, as a function of NaCl molarity at different temperatures: open circles, 95°C.; solid circles, 90°C.; open triangles, 85°C.; solid triangles, 80°C.; and open squares, 75°C.

min. and cooling at 4°C. for 1 hr. The gels were then heated. At the indicated temperature, a tube containing the gel was withdrawn from the water bath and the viscosity was measured immediately. The "melted" gels were then placed at 4°C. and were set again to obtain the original gel. Figure 9 shows the apparent viscosity of the gels heated at the indicated temperature. When cooled, the new gels had viscosities similar to those of the original gels.

Effect of Ionic Strength on Gelation

Soybean globulin dispersions (10%) containing different amounts of NaCl (0.2 to 2.0M) were heated at different temperatures, and the viscosities of the gels were measured. Figure 10 shows the results obtained. At temperatures above 70°C. the viscosities of the progel and gel decreased with increasing concentrations of NaCl. Below this temperature, the data obtained appear to be anomalous; higher viscosity is favored by higher concentrations of salts. Figure 11 also shows that only at higher temperatures (85°, 90°, 95°C.) is there a reasonably linear relation between viscosity and NaCl concentration.

Thixotropy of Gels at Various Temperatures

Figure 12 indicates changes in torque (dyne-cm.) obtained by changes in the angular velocity at which the spindle was rotated in 10% soybean globulin gels. The "T" spindle was rotated at the same position and after the maximum speed was

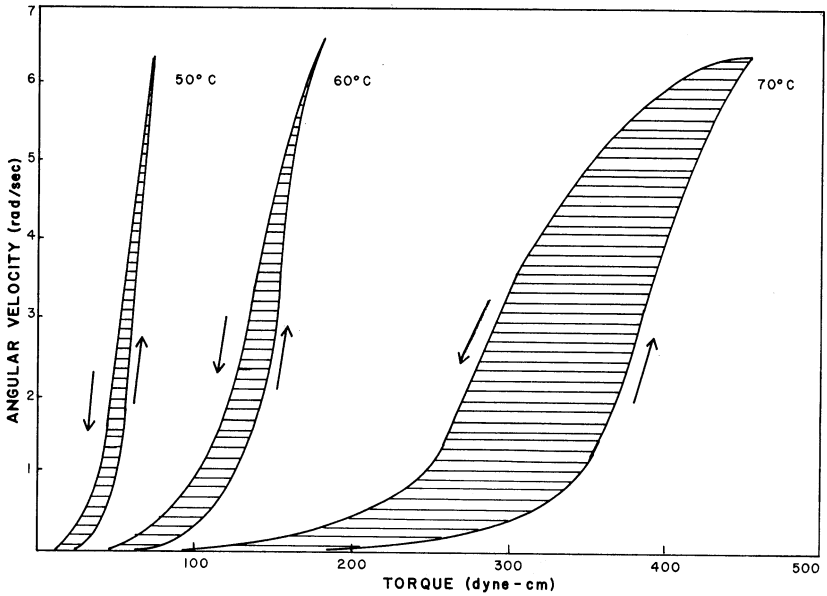


Fig. 12. Thixotropy of 10% soybean globulin gels (w./v.), pH 7.0.

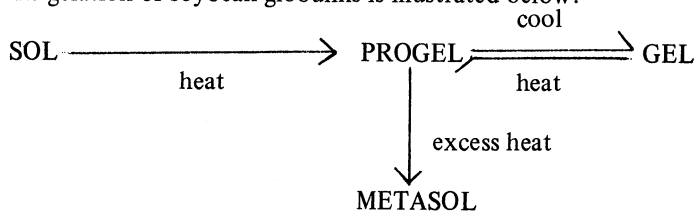
attained (in increasing steps), the rotational speed was reduced in equal steps and the torque was observed. When the data were plotted, a "hysteresis loop" was formed. The area of the hysteresis loop indicates the amount of thixotropy. It can be seen that thixotropy was increased with higher temperature of formation of the gel. However, even at rotational speeds of 60 r.p.m. (6.3 rad per sec.) the gel does not break down completely. It would be very interesting to study thixotropy in relation to a number of parameters that affect gelation. Thixotropy also shows the degree of re-formation of bonds after a mechanical breakdown of the gel.

DISCUSSION

Protein gels are formed by intermolecular interactions which produce a continuous, three-dimensional network exhibiting structural rigidity. The mechanism of formation of a gel seems to be different among the various proteins. Thus, some of the suggested mechanisms of cross-linking involve multiple hydrogen bonds (20,21), sulfhydryl-disulfide interchange (22), and formation of peptide groups (23,24). A critical parameter in gelation is the protein concentration. Below a minimum concentration, aggregation and increase in viscosity of protein solutions can be observed but no gelation occurs. It appears that an effective overlapping of the functional groups, between adjacent protein molecules or dissociated subunits, is necessary for network formation.

At concentrations between 8 and 14%, gelation of soybean globulins is accomplished by heating and subsequent cooling of the protein dispersions. The

over-all scheme of the gelation of soybean globulins is illustrated below:

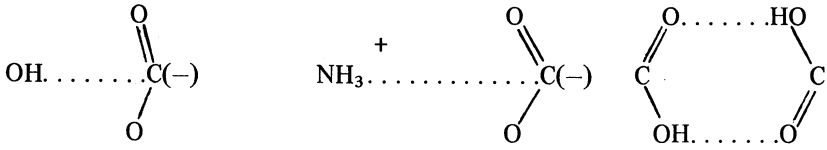


The protein dispersion (sol) is converted to a progel (which is characterized by high viscosity) by heat and then sets to a gel of still higher viscosity on cooling. The first step appears to be irreversible; that is to say, once the sol has been activated to a progel it can then only be converted into a gel or a metasol. However, the gel can be converted to the progel by heat and then cooled again to obtain the gel. Thus, the second step appears to be reversible.

The progel (or the gel) with excess heat (125°C.) forms a metasol which does not form a gel on cooling (1). A metasol is also obtained by the action of disulfide-cleaving reagents such as mercaptoethanol and sulfite, by chemical modification of functional groups, and by the action of 6M urea. It should be pointed out that the term *metasol* describes a physical state rather than a definite chemical composition.

The bonds involved in the progel-gel transition seem to be of noncovalent nature, since, depending on the protein concentration, a gel can "melt" at temperatures as low as 40°C. However, this does not exclude the possibility of some limited covalent bonding in the progel such as disulfide bridges formed by sulfhydryl-disulfide exchange, and possibly others.

The effects of pH and temperature on both the formation of the progel and the gel are very pronounced. Both the strength and maximum temperature of formation of the progel and gel are affected by pH. The gel formed by cooling is rather dependent on the multitude of hydrogen and electrostatic cross-links, because these two types of bonds are favored by lowering of temperature. The lowest gel strengths are obtained at pH values 1.0, 2.0, and 10.0. In the very acidic pH range, the carboxyl groups show minimum ionization, and at pH 10, the ionization of tyrosine is increased and that of lysine is decreased. It is possible that the decrease in the gel strength at the strongly basic pH values may be caused by inhibition of the carboxylate-phenolic group (25) (I) and carboxylate-protonated amino group (II) bonds as illustrated below:



(I) (II) (III)

However, at pH values 1.0 and 2.0 the homologous bond between protonated

carboxyl groups (24) (III) may still be formed, and at pH 10 the bond between the carboxylate group and arginine is possible. At pH 12, where the amino groups of arginine are usually titrated, there is no gel formation.

The participation of ionic bonds in the gelation phenomena of soybean globulins is suggested by the lower gel viscosities obtained in solutions of high ionic strength. However, it is possible that soybean globulins are more stable to heat-treatment in the presence of high concentrations of NaCl solutions. This stability can be visualized as an inhibition of dissociation of these proteins into subunits.

Irreversibility of the protein sol to progel state may be attributed to either one or both of two factors: (a) irreversible disruption of the quaternary structure of soybean globulins by heat, and (b) formation of covalent bonds in the progel state. However, the latter cannot be correct, because a "set" gel can be liquefied by layering solid urea on the gel. Thus, if significant covalent bonding occurred in the progel state, it should have been maintained in the gel state, and addition of urea should not liquefy the gel. In contrast, irreversible thermal disruption of the quaternary structure of glycinin, the major soybean globulin, and dissociation into subunits has been adequately documented (14,26). It is possible that similar disruption of the quaternary structure of other soybean globulins occurs with heat-treatment, since it has been shown that these components also exhibit multisubunit structures (7,12,27,28). Dissociation into subunits and unfolding of the soybean protein molecules may result in exposure of groups capable of hydrophobic bonding. The importance of hydrophobic bonds in the stabilization of the structure of soybean globulins has been reported (14,29). Hydrophobic interactions of the nonpolar groups to form an associated structural network may be involved in the formation of the progel, since this type of bonding is favored by a rise in temperature (30). At the progel stage, formation of hydrogen bonds surrounded by water are not favored because of the rise in temperature but could be formed inside nonpolar regions. However, hydrogen bonds surrounded by water may be the main source of increased viscosity observed during the progel-to-gel transition. The thermal reversibility of this transition further supports the hypothesis of hydrogen bonding.

Circle et al. (1) reported the effectiveness of low concentrations of disulfide-reducing reagents (e.g. sodium sulfite, cysteine) in depressing gelation of soybean globulins. This finding was interpreted as being indicative of participation of disulfide bonds in the gelation process. However, the mechanism of the contribution of disulfide bonds to the gelation process is not clear at present. Although low concentrations of mercaptoethanol (0.1%) inhibit gelation, high concentrations (10%) enhance it[1]. Addition of 0.1% N-ethylmaleimide, a reagent capable of blocking sulfhydryl groups and, therefore, inhibiting sulfhydryl-disulfide interchange, has no effect on gelation (see footnote 1). Similarly, Circle et al. (1) reported that cysteine at the 0.05% level inhibited gelation, but at higher concentration (0.5%) was considerably less effective. The complexity of these phenomena may be due to interplay of two types of disulfide bonds, namely, intermolecular and intramolecular. Cleavage of intermolecular disulfide bonds by

[1]Data presented at the 50th AACC Annual Meeting, Kansas City, Mo., April 1965.

low concentration of reagents may result in depression of gelation, whereas cleavage of intramolecular disulfide bridges (at high concentrations of reagents) facilitates disruption of the quaternary structure of the globulins and exposure of reactive groups due to dissociation and unfolding of the subunits, and, therefore, may result in enhancement of the gel strength.

The conversion of the progel by excess heat (125°C.) to the metasol is accompanied by chemical degradation of the proteins, since release of ammonia and decomposition of cystine have been observed. The conversion of asparagine and glutamine residues to carboxylate groups by release of ammonia may be sufficient chemical modification for obtaining a nongelling protein. This type of modification not only introduces repulsive electrostatic forces, but also greatly inhibits hydrogen-bond formations between amide groups, which may be one of the most important forces in the stabilization of soybean globulin gels.

Literature Cited

1. CIRCLE, S. J., MEYER, E. W., and WHITNEY, R. W. Rheology of soy protein dispersions. Effect of heat and other factors on gelation. Cereal Chem. 41: 157 (1964).
2. NAISMITH, W. E. F. Ultracentrifuge studies on soya bean protein. Biochim. Biophys. Acta 16: 203 (1955).
3. WOLF, W. J., and BRIGGS, D. R. Ultracentrifugal investigation of the effect of neutral salts on the extraction of soybean proteins. Arch. Biochem. Biophys. 63: 40 (1956).
4. BRIGGS, D. R., and MANN, R. L. An electrophoretic analysis of soybean protein. Cereal Chem. 27: 243 (1950).
5. SHIBASKI, K., and OKUBO, K. Starch gel-electrophoresis of soybean proteins in high concentration of urea. Tohoku J. Agr. Research 16: 317 (1966).
6. CATSIMPOOLAS, N., ROGERS, D. A., CIRCLE, S. J., and MEYER, E. W. Purification and structural studies of the 11S component of soybean proteins. Cereal Chem. 44: 631 (1967).
7. PUSKI, G., and MELNYCHYN, P. Starch-gel electrophoresis of soybean globulins. Cereal Chem. 45: 192 (1968).
8. CATSIMPOOLAS, N., LEUTHNER, E., and MEYER, E. W. Studies on the characterization of soybean proteins by immunoelectrophoresis. Arch. Biochem. Biophys. 127: 338 (1968).
9. CATSIMPOOLAS, N., and MEYER, E. W. Immunochemical study of soybean proteins. J. Agr. Food Chem. 16: 128 (1968).
10. BRIGGS, D. R., and WOLF, W. J. Studies on the cold-insoluble fraction of the water-extractable soybean proteins. I. Polymerization of the 11S component through reactions of sulfhydryl groups to form disulfide bonds. Arch. Biochem. Biophys. 72: 127 (1957).
11. WOLF, W. J., RACKIS, J. J., SMITH, A. K., SASAME, H. A., and BABCOCK, G. E. Behavior of 11S protein of soybean in acid solutions. I. Effects of pH, ionic strength and time on ultracentrifugal and optical rotatory properties. J. Am. Chem. Soc. 80: 5730 (1958).
12. ROBERTS, R. C., and BRIGGS, D. R. Isolation and characterization of the 7S component of soybean globulins. Cereal Chem. 42: 71 (1965).
13. KOSHIYAMA, I. Chromatographic and sedimentation behavior of a purified 7S protein in soybean globulins. Cereal Chem. 45: 405 (1968).
14. CATSIMPOOLAS, N., CAMPBELL, T. G., and MEYER, E. W. Association-dissociation phenomena in glycinin. Arch. Biochem. Biophys. 131: 577 (1969).
15. ELDRIDGE, A. C., and WOLF, W. J. Purification of the 11S component of soybean protein. Cereal Chem. 44: 645 (1967).
16. KOSHIYAMA, I. Purification of the 7S component of soybean proteins. Agr. Biol. Chem. 29: 885 (1965).

17. VAINTRAUB, I. Isolation of the 2S-component of soybean globulins. Biokhimiya 30: 628 (1965).
18. CATSIMPOOLAS, N., and EKENSTAM, C. Isolation of alpha, beta, and gamma conglycinins. Arch. Biochem. Biophys. 129: 490 (1969).
19. BELLO, J., BELLO, H. R., and VINOGRAD, J. R. The mechanism of gelation of gelatin. The influence of pH, concentration, time and dilute electrolyte on the gelation of gelatin and modified gelatins. Biochim. Biophys. Acta 57: 214 (1962).
20. ELDRIDGE, J. E., and FERRY, J. D. The cross-linking process in gelatin gels. III. Dependence of melting point on concentration and molecular weight. J. Phys. Chem. 58: 992 (1954).
21. BOEDTKER, H., and DOTY, P. A study of gelatin molecules, aggregates, and gels. J. Phys. Chem. 58: 968 (1954).
22. HUGGINS, C., TAPLEY, D. F., and JENSEN, E. V. Sulphhydryl-disulfide relationships in the induction of gels in proteins by urea. Nature 167: 592 (1951).
23. BELLO, J., and VINOGRAD, J. R. The biuret complex of gelatin and the mechanism of gelation. Nature 181: 273 (1958).
24. BELLO, J. The effects of biuret-complex formation and of salts on the collagen helix and on the gelation of gelatin. Biochim. Biophys. Acta 109: 250 (1965).
25. TANFORD, C., and ROBERTS, G. L. Phenolic hydroxyl ionization in proteins. I. Bovine serum albumin. J. Am. Chem. Soc. 74: 2509 (1952).
26. WOLF, W. J., and TAMURA, T. Heat-denaturation of soybean 11S protein. Cereal Chem. 46: 331 (1969).
27. CATSIMPOOLAS, N., EKENSTAM, C., ROGERS, D. A., and MEYER, E. W. Protein subunits in dormant and geminating soybean seeds. Biochim. Biophys. Acta 168: 122 (1968).
28. KOSHIYAMA, I. Chemical and physical properties of a 7S protein in soybean globulins. Cereal Chem. 45: 394 (1968).
29. FUKUSHIMA, D. Internal structure of 7S and 11S globulin molecules in soybean proteins. , Cereal Chem. 45: 203 (1968).
30. NEMETHY, G., STEINBERG, I. Z., and SCHERAGA, H. A. Influence of water structure and of hydrophobic interactions on the strength of side-chain hydrogen bonds in proteins. Biopolymers 1: 43 (1963).

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