Network Structure Formation in Thermally Induced Gelation of Glycinin

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When a 5% glycinin solution was heated at 100 °C and 0.5 ionic strength, soluble aggregates with a molecular weight of 1.8×10^6, 4×10^6, and 8×10^6 were formed at 15 s, 30 s, and 1 min, respectively, and a self-supporting gel was formed on subsequent heating. The soluble aggregates and network of heat-induced gel were visualized at the molecular level by transmission electron microscopy. The soluble aggregates were found to be in the form of strands with a thickness similar to the diameter of the native protein molecule and the network of gel seemed to be formed from the strands. The results suggest that glycinin forms gels as a result of aggregation to form strands followed by interaction of the strands to form the gel network. The presumed sequence of formation of network structure in the thermal gelation process of glycinin is presented.

The gel-forming ability of soybean proteins is of significance with respect to their usage in food systems. Extensive studies have established the basic factors affecting gelation of soybean proteins (Hermansson, 1978; Saio and Watanabe, 1978; Kinsella, 1979). The structure of protein gels, on the other hand, has been investigated by electron microscopy (Beaven et al., 1969; Burke and Rougvie, 1972; Tombs, 1974; Clark et al., 1981; Hermansson and Buchheim, 1981), giving information on the network structure and some insight into the mechanisms involved in aggregation of protein molecules to form networks. However, much less is known concerning the process of gel formation at the molecular level and the mechanisms involved in gel formation. In order to elucidate this, gelation studies on each isolated soybean globulin are highly desirable, since soybean protein is known to consist of four main components, 2S, 7S, 11S, and 15S.

The 11S globulin (referred to as glycinin), one of the major components of the soybean proteins, has the ability to form heat-induced gels (Mori et al., 1982b; Utsumi et al., 1982). In a previous paper we investigated thermal association–dissociation behavior of glycinin at an early stage of the heat-induced gelling process and demonstrated that soluble aggregates with a molecular weight of 8×10^6 are formed at 1 min of heating and subsequent heating causes formation of highly polymerized aggregates (Mori et al., 1982b). Further, at a shorter time of heating, soluble aggregates with a molecular weight of 1.8×10^6 and 4×10^6 are formed at 15 and 30 s, respectively.

In the present study, the soluble aggregates thus formed, as well as the structure of heat induced gel, were investigated by transmission electron microscopy. This report presents the sequence of formation of network structure in the thermal gelation process of glycinin.

MATERIALS AND METHODS

Materials. DEAE-Sephadex A-50 was purchased from Pharmacia Co., Ltd. 2-Mercaptoethanol, extrapure reagent, was obtained from Nakarai Chemicals (Japan). Soybeans (*Glycine max*, var. Tsuru-no-ko) were purchased from Mizuno Seed Co., Ltd. (Japan).

Preparation of Glycinin. A crude glycinin fraction was prepared from soybeans according to the method of Thanh et al. (1975). Chromatographic fractionation of the crude glycinin fraction was performed on a column of DEAE-Sephadex A-50 as described previously (Mori et al., 1979), where the column was eluted with 35 mM potassium phosphate buffer (pH 7.6) containing 10 mM 2-mercaptoethanol, 0.02% NaN_3, and NaCl in a linear gradient concentration of 0.25–0.5 M.

Heating of Glycinin. The purified glycinin in the eluting buffer was thoroughly dialyzed against 35 mM potassium phosphate buffer (pH 7.6) containing 0.4 M NaCl just before its use. After dialysis, the protein solution was adjusted to 5% protein concentration with the dialyzing buffer. Fifty microliters of the protein solution was taken in a 2.5 × 105 mm test tube covered with aluminum foil and then heated at 100 °C in a water bath for the time periods as indicated. At the end of the heating period, the test tube was removed and rapidly cooled to room temperature by immersing in tap water. In an experiment involving *N*-ethylmaleimide, 0.01 M (final) *N*-ethylmaleimide was added to the protein solution prior to heat treatment.

Method of Gelation. The 5% glycinin solution in 35 mM potassium phosphate buffer (pH 7.6) containing 0.4 M NaCl was placed in a disposable micropipet and then heated at 100 °C for 5 min to prepare gels according to the procedure as described previously (Mori et. al., 1982a).

Sucrose Density Gradient Centrifugation. The heat-treated protein solution was centrifuged on a linear sucrose density gradient (15-40% w/v). After centrifugation, the gradient was divided into 0.4-mL fractions and measured at 280 nm simultaneously with an ISCO density gradient fractionator. The details of the procedure have been described previously (Utsumi et al., 1980).

Transmission Electron Microscopy. *Negative Staining.* A solution of the protein was diluted with 35 mM potassium phosphate buffer (pH 7.6) containing 0.4 M NaCl to 0.01% protein concentration. A small drop was applied to a carbon-coated electron microscope grid and the grid was then floated on a top of a 2% solution of potassium phosphotungstate (pH 7.0). Excess liquid was drained off with a filter paper and examined in a Hitachi H-700H electron microscope.

Thin Sectioning. A small piece of glycinin gel was fixed in 2% glutaraldehyde at pH 7.2 (50 mM sodium phosphate buffer) and 4 °C for 2 h and then in 1% osmium tetraoxide at pH 7.2 (0.1 M sodium phosphate and 0.1 M sucrose) and 4 °C for 1.5 h. Ethanol dehydration was then performed using a series of increasing ethanol concentrations and the gel embedded by using a mixture of propylene oxide and epon resin. The gel block was sectioned, and thin sections were stained with uranyl acetate and lead citrate and then viewed in a Hitachi H-700H electron microscope.

Preparation of Ribosome and Tobacco Mosaic Virus. A ribosomal fraction from developing soybean seeds and a tobacco mosaic virus (TMV, strain OM) fraction

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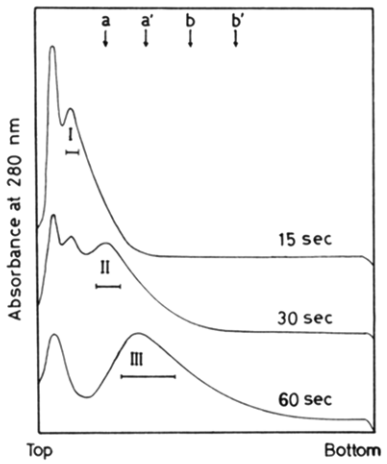

Figure 1. Sucrose density gradient centrifugation of heated glycinin. The sedimentation positions of ribosomes and TMV are indicated by arrows: a and a', monomer and dimer of ribosomes, respectively; b and b', monomeric and dimeric forms of TMV, respectively. The molecular weights of the monomeric forms of ribosomes and TMV are 4.3×10^6 and 39.4×10^6, respectively. The description of peaks I, II, and III is given in the text.

were prepared by differential centrifugation as described previously (Mori and Yokoyama, 1975).

Protein Determination. Protein was determined by the method of Lowry et al. (1951).

RESULTS

Formation of Soluble Aggregates in the Course of Gel Formation. The glycinin solutions heated for varying times were analyzed by sucrose density gradient centrifugation. As shown in Figure 1, soluble aggregates with molecular weights of 1.8×10^6 (peak I), 4×10^6 (peak II), and 8×10^6 (peak III) were formed at 15, 30, and 60 s, respectively. The molecular weights were evaluated by comparing sedimentation positions of the peaks with ribosomes and TMV. On subsequent heating, the soluble aggregate (peak III) undergoes further polymerization, resulting in formation of soluble macroaggregates and finally gel formation at 5 min and longer (Mori et al., 1982b). These soluble aggregates, as transient intermediates during gelation, seem to be stable at room temperature, since they could be isolated and their molecular weights evaluated by sucrose density gradient centrifugation. The soluble aggregates thus isolated, as well as the gel, were studied by the electron microscopy.

Electron Microscope Studies. Figure 2 shows example of the soluble aggregates visualized by microscopy at a magnification of 15000×. In the case of the soluble aggregate with a molecular weight of 1.8×10^6 (Figure 2a), short strands (~48 nm in length, arrowed) were visible; components half and less in length were also observed. In the soluble aggregate with a molecular weight of 4×10^6 (Figure 2b), straight strands twice as long (~94 nm, arrowed) as those in Figure 2a (arrowed) were apparent. Some shorter and longer strands were also seen (parts a and c of Figure 2, respectively). In the soluble aggregate with a molecular weight of 8×10^6 (Figure 2c), both branched and unbranched strands (arrowed) were visible and the strands had grown longer (170–200 nm). Parts d and e of Figure 2 show micrographs of the 5% glycinin solution heated for 2 and 4 min, respectively. Formation of network structure and its development were visible, and the impression they give is that the networks are formed from the strands observed in Fig. 2c. In the case of the soluble aggregate formed in the presence of *N*-ethylmaleimide (Figure 2f), linear strands with a length similar to those in Figure 2c were observed. Figure 3 is a micrograph of ultrathin sections of glycinin gel. the network, varying in local density, was visible at the low magnification (Figure 3a) and the network structure consisted mainly of strands (Figure 3b) similar to those seen in Figure 2e.

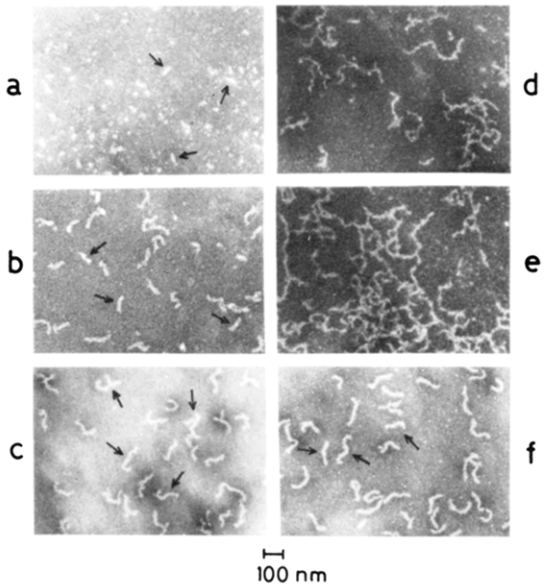

Figure 2. Transmission electron micrographs of negatively materials: (a) peak I of Figure 1; (b) peak II of Figure 1; (c) peak III of Figure 1; (d and e) unfractionated glycinin solutions heated for 2 and 4 min, respectively; (f) soluble aggretate with a molecular weight of $(4–8) \times 10^6$ obtained from the sucrose gradient centrifugation of glycinin solution heated for 10 min in the presence of *N*-ethylmaleimide. Typical strands have been indicated by arrows.

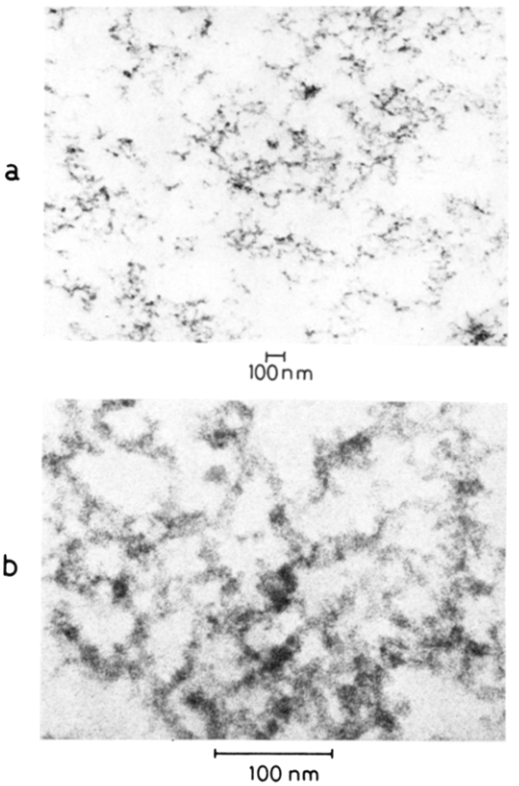

Figure 3. Transmission electron micrographs of glycinin gel: (a and b) micrographs at magnifications of 15000× and 60000×, respectively.

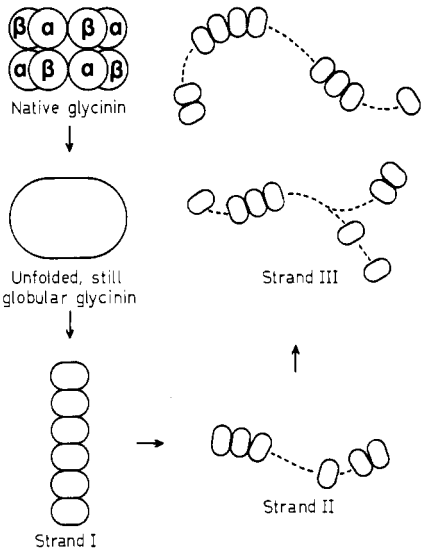

Figure 4. Schematic representation of formation of soluble aggregates in the course of gelation of glycinin. The description of α and β is given in the text. The strands II and III are represented in an abbreviated form with dashed lines.

DISCUSSION

For all of the strands (Figure 2a–c) and network constituents (Figure 2d,e), the thickness was generally found to lie in the range of 10–12 nm and was similar to the diameter of the native molecule of glycinin (hollow oblate cylinder) whose dimensions were reported by Badley et al. (1975) to be 11 × 11 × 7.5 nm. On the basis of the thickness and length of the strands, together with the molecular weights evaluated by sucrose gradient centrifugation, the simplest interpretation of the strand formation may be association of glycinin molecules, which are most likely to be only moderately unfolded and still globular. Such association may occur longitudinal positioning of one molecule on the other as illustrated schematically in Figure 4, assuming that artifacts were absent. A brief description of the proposed scheme is as follows: (1) Within a short time of heating (~15 s) short strands consisting of about six glycinin molecules are formed (strand I). (2) Strand I associates with itself to form straight strands (strand II). (3) Strand II associates with itself and/or with strand I to form both branched and unbranched strands (strand III). The gel network could then form from these strand III units. The structure of the junction zone of branched strands (strand III) is not elucidated in this study and is simiply represented by branched dashed lines in Figure 4.

The glycinin molecule is composed of 6α (acidic) and 6β (basic) subunits to form the "$\alpha_6\beta_6$" structure where α and β subunits are linked together in specific combinations through disulfide bridges with the resulting formation of intermediary $\alpha\beta$ subunits (Derbyshire et al., 1976; Kitamura et al., 1976). The 12 subunits are packed in two identical hexagons ($\alpha_3\beta_3$), placed one above the other (Badley et al., 1975). When a 5% glycinin solution was heated in the presence of *N*-ethylmaleimide, gel is not formed, while soluble aggregate is formed (Mori et al., 1982b) and found to be linear strands as shown in Figure 2f. The thickness of the strands was similar to the diameter of the native molecule of glycinin. The intermediary subunit structure ($\alpha\beta$) is retained in this soluble aggregate in which the intermediary subunits are bound together by noncovalent forces that are severed by sodium dodecyl sulfate (Mori et al., 1982b). Thus, is seems that on heat treatment glycinin tends to associate together through possibly hydrophobic interaction to form linear strands while retaining its substructure, pairing of the acidic and basic subunits.

On the other hand, it has been demonstrated that glycinin contains at least 20 disulfide and 2 sulfhydryl groups per mole of protein (Draper and Catsimpoolas, 1978). In the course of gelation of glycinin by heating through formation of the strands of various lengths and network, intermolecular disulfide exchange reactions proceed and new disulfide bonds are most likely to be formed. Therefore, in the occasion of gel formation, glycinin may associate together by intermolecular disulfide bonds as well as through hydrophobic interaction to form the strands. The soluble aggregates formed in the presence of *N*-ethylmaleimide and those formed in its absence seem to be similar except for the presence of branched strands in the latter (Figure 2c,f); however, they are likely to be different from each other with respect to intermolecular disulfide bondings. The disulfide bridge formation through intermolecular disulfide exchange reactions may participate in the formation of strands, particularly branched ones, followed by subsequent network formation and gel formation, where the sulfhydryl groups may play an important role as a trigger that sets off the disulfide exchange reactions.

Recently, Hermansson and Buchheim (1981) have investigated the structure of soy protein gels by transmission electron microscopy. They observed a network structure consisting mainly of fine strands, an orientation on the molecular level in the form of strands and fine ring structure. Our results are consistent with and substantiate their findings and suggest that glycinin, one of the major components of soy protein, contributes significantly to the network structure of soy protein gels. Clark et al. (1981) have studied the structure of thermally induced globular protein gels by electron microscopy. They observed filamentous strands constituted network structure and proposed a "string of beads" model for the aggregation process, involving only moderately unfolded, and still globular, protein molecules. This model seems to hold well here, except that a "bead" corresponds to a glycinin molecule and not to its constituent subunit.

The process of network formation in thermally induced gelation of proteins may be considered to be an artificial aggregating system. However, regularity in size of each strand as a transient intermediate in the formation of network of heat-set gel of glycinin under the conditions used here suggests the involvement of an ordered mechanism: The heat treatment may have caused certain changes on the surface of glycinin molecule, stimulating enhanced and specific aggregation, thereby resulting in the formation of regular strands. Sites for noncovalent interactions, possibly hydrophobic interaction, and disulfide exchange on the glycinin molecules may become more available and accessible, thereby facilitating the association reaction of glycinin molecules, resulting in the formation of strands and networks. Further investigation is required in order to elucidate the mechanisms involved in the formation of regular strands, branched strand, and networks from the strands.

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Fluorometric Determination of Total Vitamin C and Total Isovitamin C in Foodstuffs and Beverages by High-Performance Liquid Chromatography with Precolumn Derivatization

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A reliable and sensitive high-performance liquid chromatographic (HPLC) method is described for the simultaneous determination of total vitamin C (*l*-ascorbic acid, AA, plus dehydro-1-ascorbic acid, DHAA) and its C-5 epimer, total isovitamin C (erythorbic acid, EA, plus dehydroerythorbic acid, DHEA), in foodstuffs and beverages. After extraction AA and EA are oxidized enzymatically to DHAA and DHEA with the aid of ascorbate oxidase (EC 1.10.3.3). The latter compounds are condensed with *o*-phenylenediamine (OPDA) to their highly fluorescent quinoxaline derivatives. These derivatives are separated on a reversed-phase HPLC column and detected fluorometrically. Total vitamin C and isovitamin C can be determined in concentrations as low as 0.2 μg/g. The amounts of DHAA and DHEA present in foodstuffs and beverages can be determined separately by the same procedure with omission of the enzymatic oxidation.

Vitamin C, a water-soluble vitamin, consists of AA and its oxidized form DHAA. Both forms are equally biologically active (Procházka, 1964). The C-5 epimer of AA, erythorbic acid, which is much less biologically active (Procházka, 1964; Pelletier and Godin, 1969), may be used as an antioxidant in foods and beverages.

Numerous methods for the analysis of vitamin C and/or isovitamin C in foodstuffs have been described. These include the indicator-dye reduction method with dichlorophenolindophenol (Hiromi et al., 1980), the ketone derivatization method with dinitrophenylhydrazine (Bourgeois and Mainguy, 1974; Pelletier and Brassard, 1977), fluorometric methods by condensation of DHAA with OPDA (Kirk and Ting, 1975; Egberg et al., 1977), an enzymatic method using ascorbate oxidase (Beutler and Beinstingl, 1980), and HPLC methods with UV detection (Geigert et al., 1981; Bui-Nguyên, 1980; Dennison et al., 1981; Keating and Haddad, 1982) and electrochemical detection (Pachla and Kissinger, 1976; Rückemann, 1980).

However, the indicator-dye reduction method, the ketone derivatization method and the fluorometric methods are not very specific and do not differentiate between AA and EA. Furthermore they have the drawback that blank values have to be determined owing to chemical interference in the color-inducing reaction that can be a source of error. The enzymatic method is difficult to perform in large-scale routine analysis and also does not differentiate between AA and EA.

HPLC methods with electrochemical detection only allow the determination of the reduced forms, AA and EA. Dennison et al. (1981) described an HPLC method for the analysis of total vitamin C in beverages by UV measurement of AA after reduction of DHAA with homocysteine. Keating and Haddad (1982) and Wimalasiri and Wills (1983) described HPLC methods with UV detection for the simultaneous determination of AA and DHAA. However, the authors mentioned did not consider possible interference by isovitamin C.

Therefore, we developed an HPLC method for the simultaneous determination of total vitamin C and isovitamin C. After enzymatic oxidation of AA and EA to DHAA and DHEA, the latter, having themselves insufficient UV absorptivity, are condensed with OPDA to their highly fluorescent 3-(1,2-dihydroxyethyl)furo[3,4-*b*]-quinoxalin-1-ones (DFQ and IDFQ, respectively). These derivatives are separated on a reversed-phase HPLC column and detected fluorometrically.

Furthermore, by omission of the enzymatic oxidation step, the concentrations of DHAA and DHEA in foodstuffs

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