Gelation of Edible Blue-Green Algae Protein Isolate (*Spirulina platensis* Strain Pacifica): Thermal Transitions, Rheological Properties, and Molecular Forces Involved

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Proteins isolated from blue-green algae *Spirulina platensis* strain Pacifica were characterized by visible absorption, differential scanning calorimetry (DSC), viscometry, and dynamic oscillatory rheological measurements. Unique thermal unfolding, denaturation, aggregation, and gelation of the algal protein isolate are presented. DSC analysis showed that thermal transitions occur at about 67 and 109 °C at neutral pH. Calcium chloride stabilized the quaternary structure against denaturation and shifted the transitions at higher temperatures. Viscometric studies of *Spirulina* protein isolate as a function of temperature showed that the onset of the viscosity increase is closely related to the dissociation−denaturation process. Lower viscosities were observed for the protein solutions dissolved at pH 9 due to an increased protein solubility. Solutions of *Spirulina* protein isolate form elastic gels during heating to 90 °C. Subsequent cooling at ambient temperatures caused a further pronounced increase in the elastic moduli and network elasticity. *Spirulina* protein isolate has good gelling properties with fairly low minimum critical gelling concentrations of about 1.5 and 2.5 wt % in 0.1 M Tris buffer, pH 7, and with 0.02 M $CaCl_2$ in the same buffer, respectively. It is suggested that mainly the interactions of exposed hydrophobic regions generate the molecular association, initial aggregation, and gelation of the protein isolate during the thermal treatment. Hydrogen bonds reinforce the network rigidity of the protein on cooling and further stabilize the structure of *Spirulina* protein gels but alone are not sufficient to form a network structure. Intermolecular sulfhydryl and disulfide bonds were found to play a minor role for the network strength of *Spirulina* protein gels but affect the elasticity of the structures formed. Both time and temperature at isothermal heat-induced gelation within 40−80 °C affect substantially the network formation and the development of elastic modulus of *Spirulina* protein gels. This is also attributed to the strong temperature dependence of hydrophobic interactions. The aggregation, denaturation, and gelation properties of *Spirulina* algal protein isolate are likely to be controlled from protein−protein complexes rather than individual protein molecules.

Keywords: *Spirulina platensis; algae protein; thermal transitions; denaturation; aggregation; gelation; molecular forces; heating rate*

INTRODUCTION

Aquatic algae, a genetically diverse group of organisms, are attractive natural sources of bioactive molecules. As autotrophes they require only light, carbon dioxide, and inorganic nutrients to sustain growth. Food use of algae biomass may have technical and commercial advantages (*1a*, *2*−*5*). The seaweed phycocolloids carrageenans, agar, and alginate are already widely used in foods as gel-forming or thickening agents. However, proteins from aquatic algae have so far received much less attention.

Marine and freshwater algae vegetables are potentially good sources of proteins (*1a*, *6*−*9*). Higher levels of proteins were found in green (Chlorophyceae) and blue-green algae (Cyanobacteria), generally 40−65% of dry weight. The latter values and the nutritive content of algae proteins are comparable and in many cases superior to that of most conventional protein feed supplements (*5*, *10*).

Spirulina are blue-green algae (*Cyanobacterium*) belonging to the family Oscillatoriaceae and form unbranched, multicellular helicoidal filaments with a length of ∼200−300 μm and and a breadth of 5−10 μm (*11*). *Spirulina* has a high protein content, 60−70 wt %, and high productivity with a photosynthetic conversion rate of 8−10% compared to only 3% in most terrestrial plants (e.g., soybeans). The amino acid composition of *Spirulina* protein is generally well balanced, reflecting its potential as human food and as a source of natural products (*6*, *12*, *13*). The amino acid concentrations show as well that *Spirulina* protein is low in sulfur-containing amino acids and in tryptophan (*12*). The ratio of acidic (aspartic and glutamic acid) to basic (arginine, lysine) residues present in the protein reveals that *Spirulina* is mildly acidic and carries a negative charge at neutral pH.

There is very little detailed information available on the functional properties of *Spirulina* products. In a recent study we have focused on the surface activity of proteins and lipid components isolated from *Spirulina* algae to investigate their capability to stabilize emulsions, foams, and dispersions (*1b*). The proteins of

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Spirulina blue-green algae are quite capable of reducing the interfacial tension at the aqueous/air interface already at relatively lower bulk concentrations than common food proteins. We observed that the surface active components are likely to be protein and/or protein−pigment complexes rather than individual protein molecules. It was as well possible to separate out fractions with different colors (blue, dark blue, blue-green, and green) by ultracentrifugation, and these fractions have different interfacial properties. Moreover, the SDS-PAGE gel of the protein isolate sample features several bands with corresponding molecular weights in the range 14.4−116 kDa, with the most pronounced bands corresponding to molecular weights of 20.1 and 43 kDa (*1b*). The proteins isolated from the *Spirulina* algae have isoelectric points between 2.8 and 3.5. Previous studies have also been concerned with the water and fat absorption, emulsification capacity, foaming capacity, and stability of *Spirulina* flour and protein concentrate from algal cells and compared with that of soybean meal and egg protein (*14*, *15*).

Gelation is another important functional property of proteins, which can be a definite factor for the potential use of a protein-rich material in food (*16*). Many proteins gelate during or after simple heating, which unfolds the polypeptide chains and unmasks some of the reactive groups involved in the cross-linking. Moreover, the protein gelation often involves a denaturation or conformational alteration of the native protein followed by aggregation of the formed coils to build up the gel matrix (*17*). The exact mechanism of gel formation will strongly depend on the protein and the conditions. Studies have shown that specific conditions, such as protein concentration, pH, ionic strength, reducing agents, denaturants, and water miscible solvents, all have profound influence on the nature of the gelation of proteins (*18*−*21*). Salts, for example, alter the native conformation of food proteins and affect their temperature of denaturation (*22*). In solution, ions exert their influence by affecting the net charge of proteins and hydration and electrostatic interactions (*23*). Sugars and polyols stabilize proteins against heat denaturation by increasing the structure of water, which indirectly strengthens hydrophobic interactions and stabilizes protein conformation (*24*). The results of those studies have led to the general conclusion that protein gelation takes place under conditions that allow ordered interchain, intermolecular, or interparticle interactions usually through hydrogen bonds, hydrophobic bonds, electrostatic interactions, and disulfide linkages between polypeptides.

The objectives of this study were to investigate the thermal dissociation−association and the structural−functional properties of *Spirulina* protein isolate using the combination of visible absorption spectrometry, differential scanning calorimetry (DSC), viscometry, and dynamic mechanical analysis. Visible absorption is a sensitive probe to characterize the composition and the conformation of pigment−protein fractions and has been utilized extensively in algal biosystems (*25*). DSC has been established as a technique for studying thermal denaturation and conformational transition of proteins and is used frequently in the study of various food protein systems (*22*). Dynamic oscillatory provides a relatively simple and convenient method for characterizing protein gelling, and several systematic studies have been reported (*26*−*29*). Viscosity measurements can relate functional properties of proteins to a physical property and provide an additional tool for process monitoring and control. We also compare the gelation and related structural−functional properties of *Spirulina* protein isolate in different environments to derive the molecular forces involved and the kinetics of the heat-induced gelation processes. Such algae protein characteristics have received up to now no attention in the literature. To the best of our knowledge this is first study to show that it is possible to create structures (gels) from a thermally denatured algae protein, and thus new scientific and technological directions have opened for the application of algae proteins. A preliminary account of this work has been published previously (*30*).

MATERIALS AND METHODS

Materials. *Chemicals.* All chemicals described below were of analytical reagent grade and were obtained from Sigma Chemical Co..

Extraction Procedure of the Spirulina Protein. Spray-dried algal powder of *Spirulina platensis* strain Pacifica (lot P004111) was kindly donated from Cyanotech Corp. (Kailua-Kona, HI). The typical general composition as reported from Cyanotech Corp. is protein, 60%; carbohydrates, 19%; lipids, 6%; minerals, 8%; and moisture, 7%.

The *S. platensis* strain Pacifica protein isolate has been extracted from the algal powder by dissolution of the algae under reductive conditions in NaOH, where previous studies have shown greater solubility (*14*). The soluble protein then was separated by acidic precipitation and freeze-dried as described elsewhere (*1a*, *30*). Our extraction procedure involved dissolution of algal powder in dilute alkali (pH 10) and centrifugation at 25 °C and 7000 rpm (9039*g*) followed by supernatant collection (supernatant A). The pellet was collected and redissolved again in alkali followed by new centrifugation at 25 °C and 7000 rpm and supernatant collection (supernatant B). Both supernatants A and B were adjusted at pH 3 with 0.1 M HCl to precipitate the protein. The precipitate was recovered by centrifugation (7000 rpm for 30 min, 25 °C), and the pellet was neutralized (0.01 M NaOH) and freeze-dried. The freeze-dried protein was dissolved in Millipore water, dialyzed for 2 days at 5 °C against Millipore water to eliminate the presence of salt, and centrifuged at 7000 rpm for 30 min to remove any particulate matter; the pH was then adjusted to 7, and the protein was freeze-dried again. The freeze-dried powder was defatted with *n*-hexane (three times over a period of 5 h, 5 °C), dried under vacuum in a desiccator, and stored at 5 °C for further analysis. The nitrogen content of *Spirulina* was determined according to the micro-Kjeldahl method (a factor of 6.25 was used to convert nitrogen to protein). *Spirulina* algae and the protein isolate were found to contain 59.1 and 78.6% protein, respectively. Note that proteinous material isolated from *Spirulina* algae contains, apart from a complex mixture of proteins, pigments that are covalently linked to some of the proteins. These protein−pigment components are supramolecular complexes in which the pigment is tightly bound to the algae protein, and they are metabolically active (*31*, *32*).

All protein solutions were freshly made each day and used immediately before the tests. All solutions used for the rheological and visible absorbance measurements were previously centrifuged gently to remove air bubbles.

Measurements. *Visible Absorption. S. platensis* strain Pacifica protein isolate (0.5 mg/mL) was dissolved in 0.1 M Tris-HCl buffer, pH 7, and in the same buffer containing 0.02 M $CaCl_2 \cdot 2H_2O$. The solutions were placed in a Perkin-Elmer Lambda 14 UV−vis spectrometer with UV Winlab software.

Calorimetric Measurements. DSC measurements were made using a Seiko DSC 6200 calorimeter from Seiko Instruments Inc. (Nordlab, Sweden) at a scan rate of 10 °C/min covering the temperature range from 30 to 180 °C. Coated sample pans

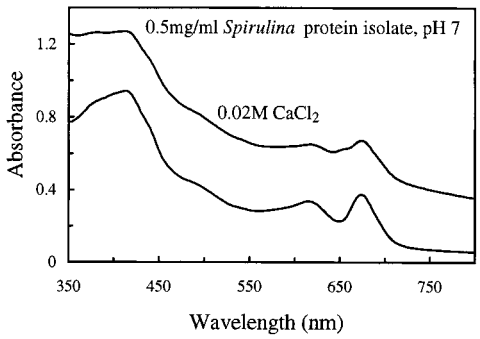

Figure 1. Absorbance spectrum of 0.5 mg/mL *S. platensis* strain Pacifica protein isolate at 25 °C, in 0.1 M Tris-HCl buffer, pH 7, and in 0.02 M $CaCl_2 \cdot 2H_2O$ in 0.1 M Tris-HCl buffer, pH 7.

of aluminum from TA Instruments were used. DSC pans were filled with ~4 mg of the protein isolate powder, and 20 mg of the buffer solution was added using a micropipet. For the studies of the pH and the ionic strength, *Spirulina* protein isolate was prepared in 0.1 M Tris-HCl buffer solution at pH 4.5, 7, and 9 and in 0.1 M Tris-HCl buffer at different pH values containing 0.004 and 0.02 M $CaCl_2 \cdot 2H_2O$. To study the effect of sugars, *Spirulina* protein was prepared in 0.1 M Tris-HCl buffer, pH 7, containing 30 or 50 wt % sucrose. A reference pan was adjusted to ±0.1 mg of the same weight using each buffer solution. The pans were hermetically sealed and weighed accurately.

Viscometry. Spirulina protein isolate (2 wt %) was dissolved in 0.01 M sodium phosphate buffer, pH 7.5, 9, and 5, containing 50 mM NaCl. The specific viscosities of the protein solutions were determined using a Schott Geräte capillary viscometer (Schott Geräte) immersed in a Haake thermostated bath. The capillary was connected to a Schott Geräte AVS 440 electronic device that determined the flow time and the relative viscosity of the solutions. The flow times of the buffer solution at 25 and 70 °C were about 180 and 90 s, respectively. All of the samples (volume of 20 mL) were left for 30 min to equilibrate at each measured temperature prior to any viscosity monitoring.

Low-Amplitude Oscillatory Measurements. Rheological measurements under low-amplitude oscillatory shear were performed on a controlled stress Carri-Med CSL100 rheometer (TA Instruments) using a parallel plate geometry (40 mm radius; 1 mm separation) at a frequency of 1 Hz. All measurements were performed with 2% strain because strain sweeps on selected gels demonstrated that the working deformation was well within the linear viscoelastic region. Solutions were loaded on the rheometer at 30 °C, and structural changes were monitored on heating from 30 up to 90 °C at 1 °C/min before subsequent cooling, also at 1 °C/min, to 5 °C. Cooling scans were followed by a frequency sweep between 0.01 and 10 Hz. For the isothermal heating, *Spirulina* protein isolate solutions were placed on the rheometer plate that had been preheated at different temperatures, between 40 and 80 °C. The above sequence of experimental procedures allowed recording of storage modulus (G'), loss modulus (G''), tan δ ($=G''/G'$), and complex viscosity [$\eta^* = (G'^2 + G''^2)^{1/2}/\omega$] as functions of time, temperature, and frequency of oscillation ω. The sample periphery was coated with silicone oil to minimize loss of solvent or adsorption of atmospheric moisture.

RESULTS AND DISCUSSION

Protein−Pigment Complexes in *Spirulina*. The absorption spectrum of the dominant protein−pigment complexes in *Spirulina* protein isolate is shown in Figure 1. The absorption spectrum of *Spirulina* protein isolate is characterized by maxima at 674, 620, and 410 nm corresponding to the presence of allophycocyanin−, phycocyanobilin−, and chlorophyll−protein pigments, respectively. Richmond (*33*) suggested that the two phycobiliproteins, c-phycocyanin and allophycocyanin, normally comprise quantitatively ~20% of cellular *Spirulina* protein. The presence of divalent ions (0.02 M $CaCl_2$) increased the turbidity and the absorbance of the *Spirulina* protein solution but did not produce conformational changes or modify the environment of the protein−pigment complexes (Figure 1). The increased light scattering when salt was added was probably due to the decrease of the protein solubility and formation of aggregates. This is in accordance with the studies of Crespi et al. (*34*), who observed that in the presence of salts the solubility of *Spirulina* protein decreased, unlike that of other proteins. They supposed that, as the proteins are present as pigment−protein complexes, the force of attraction between the protein ion and the salt is less, which may account for the lower solubility.

When the pH was adjusted to 12, or when 4 M urea was added, a similar spectrum with a lower absorbance value was obtained, whereas the maximum absorbance at 620 nm was substantially diminished (data not shown). This may suggest oxidation of the protein−pigment complexes at highly alkaline environments and at high molar concentrations of protein denaturing agents. After initial heating of the protein solution at 80 °C for 1 h and then measuring the absorbance at 25 °C, the maximum at 620 nm also was diminished, both in buffer and in the presence of $CaCl_2$.

Calorimetry−Thermal Denaturation. The thermal denaturation of proteins can often induce pronounced endothremic peaks on the calorimerty. Such heat-induced conformational transitions were also observed in the present protein. The thermograms of *Spirulina* protein isolate exhibited two main endothermic peaks with midpoint transition temperatures at ≈67 and ≈109 °C in 0.1 M Tris-HCl buffer at pH 7 (Figure 2a). When the protein isolate was reheated, a broad endothermic peak was obtained that covers the above transitions and denotes a partially denatured state and absence of reversibility. These thermal transitions may originate from the two major components with $S_{20,w}$ values of 2.6 and 4.75 S detected from the sedimentation velocity pattern in *S. platensis* (*15*). At pH 9 the thermal denaturation temperature of the first peak was decreased by almost 7 °C. This is probably due to the increase in protein solubility, which led to dissociation of the protein aggregates and decrease of the denaturation temperature. At pH 4.5 in buffer solution almost no differences were observed for the midpoint transition temperatures (results not shown). However, the enthalpy was decreased, probably due to protein aggregation because the solubility was decreased; aggregation can be considered to be an exothermic reaction.

When 0.004 or 0.02 M $CaCl_2$ was added (Figure 2b,c), both peak transition midpoint temperatures followed the same dependence with pH as without salt but were, nevertheless, progressively increased. At pH 9 the denaturation transitions were influenced most. Therefore, $CaCl_2$ has a stabilizing effect on the quaternary structure against dissociation and denaturation and shifts the transitions to higher temperatures than in the case of buffer solution. At higher salt concentrations (0.02 M) a small transition was obtained above 125 °C, but there are no clear conclusions at present from these structural states of the protein. It is possible that the high salt concentration promoted protein−protein aggregation after denaturation.

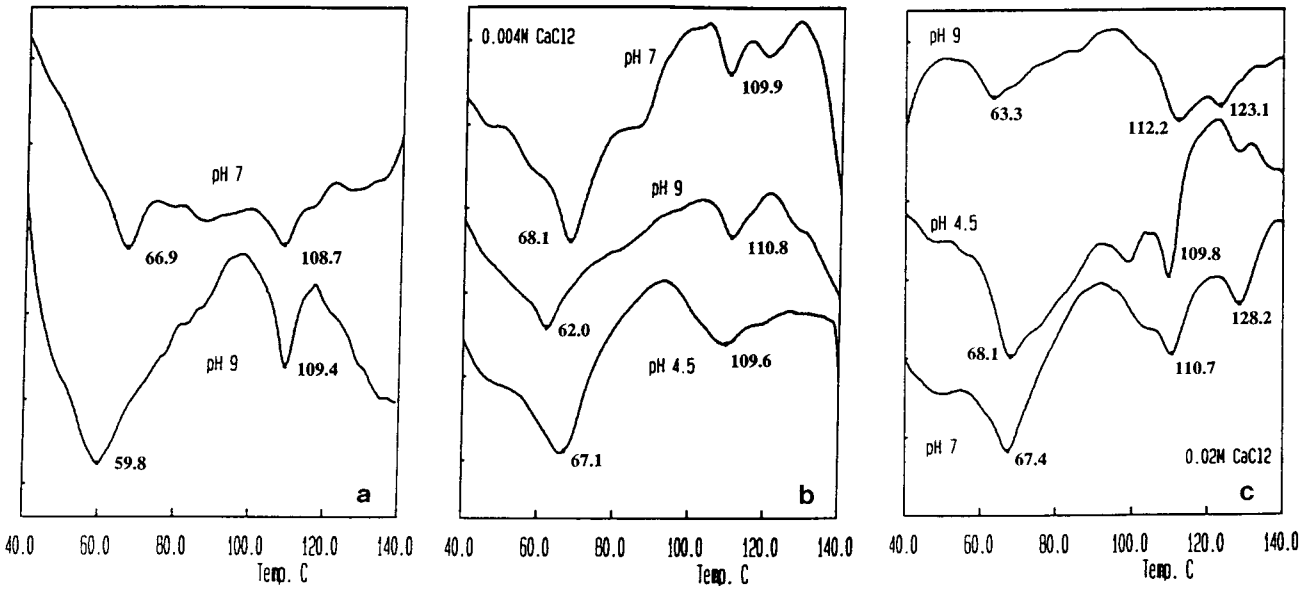

Figure 2. DSC thermograms of the denaturation of *S. platensis* strain Pacifica protein isolate: (a) in 0.1 M Tris-HCl buffer; (b) in 0.004 M $CaCl_2 \cdot 2H_2O$ at pH 4.5, 7, and 9; (c) in 0.02 M $CaCl_2 \cdot 2H_2O$ in 0.1 M Tris-HCl at pH 4.5, 7, and 9. Heating rate was 10 °C/min. Scale is in 0.05 mW.

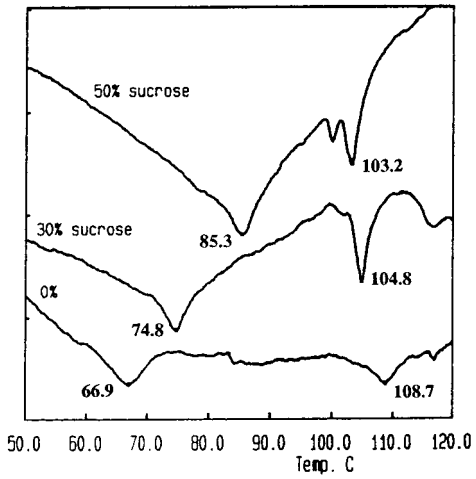

Figure 3. DSC thermogram of the denaturation of *S. platensis* strain Pacifica protein isolate in 0.1 M Tris-HCl buffer, pH 7, and at 0, 30, and 50 wt % of sucrose. Heating rate was 10 °C/min. Scale is in 0.1 mW.

In the following section we address the effects of adding sucrose to the aqueous protein solution. It is known that polyhydric cosolvents such as sugars stabilize the structure of the proteins against denaturation and strengthen the hydrophobic interactions. Two types of hydrophobic interactions, which are influenced in different ways by added solutes, have been proposed (*24*). Pairwise interactions in which hydrophobic groups associate but otherwise remain surrounded by water, and complete transfer of a hydrophobic group from an aqueous to a nonpolar environment. Sucrose was found to reduce the tendency for a complete transfer of hydrophobic groups from an aqueous to a nonpolar environment (*24*). As shown in Figure 3, the magnitude of the stabilizing effect varies progressively with the amount of sucrose added. The first transition temperature of the protein was shifted to higher temperature (from 67 to 75 and 86 °C, with 0, 30, and 50 wt % sucrose, respectively). Nevertheless, the second transition temperature was unexpectedly decreased. It is probable that these transitions are a consequence of the reaction between the protein and a reducing sugar (Maillard reaction). The Maillard reaction is in principle a second-order chemical reaction (*35*); thus, a significant effect of the heating rate on the midpoint temperature of the second transition could explain the effect of sugars at high temperatures. Further studies using different heating rates are essential to understand this behavior.

Temperature and Concentration Dependence on the Viscosity of *Spirulina* Protein Isolate. In addition to the calorimetric data, evidence for the protein thermal transitions and protein−protein intermolecular associations can be provided from viscosity measurements. The viscosity (Figure 4a) of *Spirulina* protein isolate was decreased when the temperature was increased, as observed in most proteins. However, beyond 50 °C in addition to the decrease in viscosity due to the increase in kinetic energy, the thermal denaturation contributed to the rheology of the protein solution and the viscosity was increased (Figure 4a). Most probable is that as the protein unfolds or uncoils, the axial ratio and hydrodynamic volume increased and the change in conformation led to higher viscosity. Overall, *Spirulina* protein is sensitive to temperature and an irreversible denaturation can occur and alter the viscosity at temperatures as low as ≈60 °C, in accordance with the above calorimetric data. The decrease and subsequent increase of the viscosity of *Spirulina* protein isolate within the temperature range between 10 and 50 °C and above 50−70 °C followed an Arrhenius type of dependence. The energies of flow activation, calculated from a plot of the log of viscosity versus $1/T$ (where T is the absolute temperature), were 2.2 and 7.7 kJ/mol·K, for the low- and high-temperature ranges, respectively.

Moreover, the changes in viscosity at various pH conditions are obviously of practical importance to the stability and processing of the *Spirulina* protein dispersions and mainly could be related to the changes in particle size. Figure 4b illustrates the viscosity response of the *Spirulina* protein isolate concentrations at pH 7.5 and 9. Lower viscosities were observed for the protein samples dissolved at pH 9 buffer solution. Upon dissolution of the protein at pH 9 the solubility was

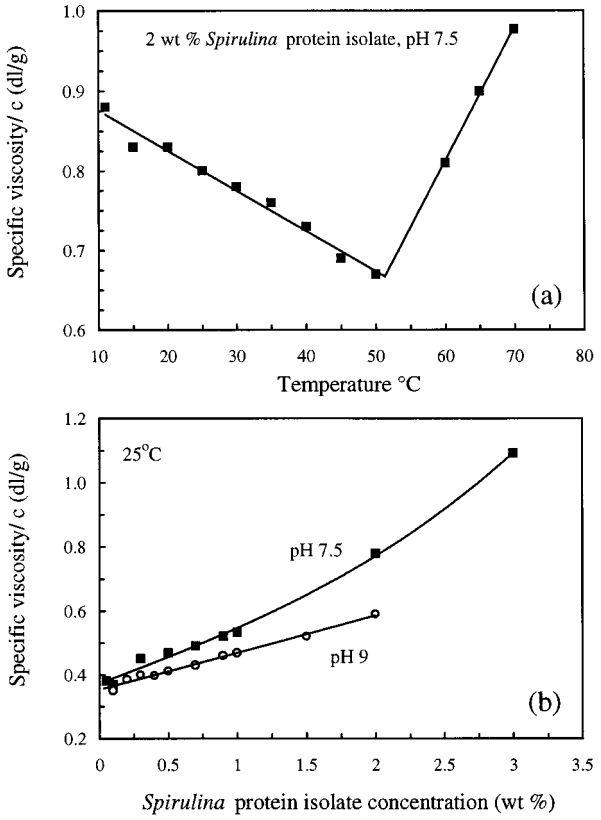

Figure 4. (a) Changes in the specific viscosity of *S. platensis* strain Pacifica protein isolate with temperature. 2 wt % *Spirulina* protein isolate in 0.01 M sodium−phosphate buffer, 0.05 M NaCl, pH 7.5. (b) Changes in the specific viscosity as a function of *S. platensis* strain Pacifica protein isolate concentration at pH 7.5 and 9 in 0.01 M sodium phosphate buffer, 50 mM NaCl.

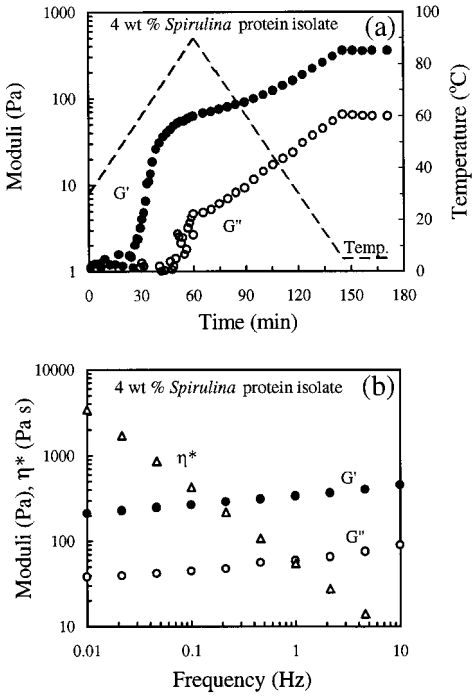

Figure 5. (a) Changes of G' (●) and G'' (○) during heating of a 4 wt % solution of *S. platensis* strain Pacifica protein isolate from 30 to 90 °C and then to 5 °C at a rate of 1 °C/min. Dotted line represents the temperature history. (1 Hz, 2% strain.) (b) Changes of G' (●), G'' (○), and complex viscosity η^* (△) as a function of frequency of oscillation of a 4 wt % solution of *S. platensis* strain Pacifica protein isolate at 5 °C. (2% strain.)

increased (*14*), and thus as the protein aggregates were dispersed into smaller particles at these high soluble conditions, the sediment volume and the viscosity will be obviously decreased. At pH ∼4.5, which is closer to the isoelectric point (3.5), the viscosity was higher by a factor of ∼1 than at the neutral pH; however, more erratic values were obtained (results not shown). Most probably this is related to the changes (increase) in particle size because the solubility of *Spirulina* protein isolate at pH 4.5 was decreased, and the protein tends to form aggregates that include the core, which is not accessible for maximum hydration.

Gelation of *Spirulina* Protein Isolate. We further show that gels based on physical association can be obtained from algae protein aqueous solutions. The network formation of *Spirulina* protein isolate was examined by dynamic viscoelastic measurements. Figure 5a illustrates the thermorheogram of 4 wt % *Spirulina* protein isolate, where clearly the characteristic sigmoidal modulus−temperature profile (*36*) of the cooperative gelation of native globular proteins does occur. The structures obtained for *Spirulina* protein isolate below the denaturation temperature range (temperature between 30 and ≈60 °C) were reversible with no thermal hysteresis on subsequent cooling. Above ≈60 °C denaturation proceeds and reinforces the modulus of *Spirulina* protein networks; however, now the association of aggregates results in an irreversible network structure. The internal rearrangements and structure of *Spirulina* gels seem to settle within the experimental time scale of an hour at 90 °C, as indicated by the moduli traces that asymptotically approach equilibrium values (data not shown). This denotes that the heating treatment was sufficient to complete the heat-induced aggregation of *Spirulina* protein isolate. During long curing at 90 °C for some hours (and mainly at pH 9) the interaggregate adherence decreased, which can be attributed to the stability of the hydrophobic forces (as will be shown below). From the present oscillation measurements the functional consequences related to the second denaturation peak observed by calorimetry at temperatures around 110 °C are not clear. Furthermore, on cooling a progressive increase in elastic modulus was observed (Figure 5a), which is usually correlated with the formation of multiple hydrogen bonds and with the diminishing entropic drive of the system, that allowed the development of short-range, noncovalent interactions between aggregated molecules. The nature of the molecular forces involved in the gelation of *Spirulina* protein is discussed below. Briefly, however, it is firmly established that dissociation into subunits and unfolding of the protein molecules as temperature increases generally result in exposure of reactive groups capable of protein−protein hydrophobic bonding. This leads indeed to an association of the protein aggregates, which form the points of physical cross-linking. Moreover, other electrostatic and hydrogen-bonding interactions are also favored; these cross-linking interactions, as well as disulfide−sulfhydryl interchange reactions, are primarily responsible for the subsequent aggregation and gelation of globular proteins (*37*). Figure 5b illustrates the response of the same sample to increasing small-amplitude angular frequency (ω). A gellike response (G' is substantially higher than G'') is obtained with only slight variation of frequency dependence on both moduli and complex viscosity. In terms of complex dynamic viscosity the logarithmic

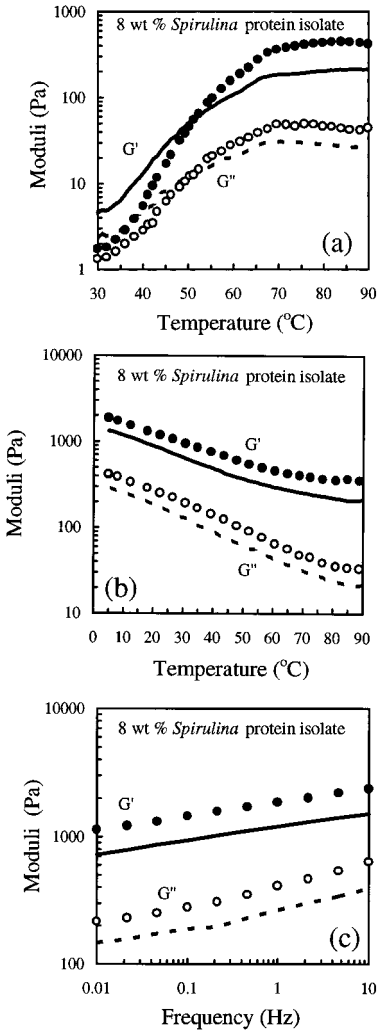

Figure 6. Dynamic oscillatory measurements of 8 wt % *S. platensis* strain Pacifica protein isolate at 1 Hz, 2% strain, and 1 °C/min in 0.1 M Tris-HCl buffer and in 0.02 M $CaCl_2 \cdot 2H_2O$ in 0.1 M Tris-HCl buffer and pH 7, as a function of temperature (a) during heating and (b) during cooling and (c) as a function of frequency of oscillation at 5 °C: G' (●), G'' (○) in Tris buffer; G' (—), G'' (- - -) in $CaCl_2$. (1 Hz 2% strain.)

power law dependence of η^* versus frequency is essentially linear, with no indication of a leveling off to a horizontal "Newtonian plateau" at low frequency, and has a slope of −0.9. This value is close to the limiting shear rate dependence of viscosity for true gels (−1) (*38*).

The onset of gelation (defined as $G' > G''$ at the frequency of 1 Hz) is heavily governed by the concentration, and generally at concentrations below 8 wt % after a "lag period", G' increases as soon as the heating commences (cf. Figure 5a). The gelation temperature decreased as the protein concentration was increased, and thus for 2, 4, and 6 wt % protein isolate the gelation occurs at 60, 52.5, and 43 °C, respectively. However, at concentrations beyond 8 wt % structures were also observed ($G' > G''$ at the frequency of 1 Hz) at the first stage of thermal gelation with low moduli values (Figure 6a,b). As is evident, formation of a protein coagulum can result by heating the *Spirulina* protein isolate below the denaturation temperature. Although it is commonly assumed that a protein denatures before it aggregates and forms a gel (*39*), another way to form a network is by direct aggregation, and so thermal gelation of some proteins occurs at temperatures well under the temperature of denaturation (*28*, *40*). This has been taken as

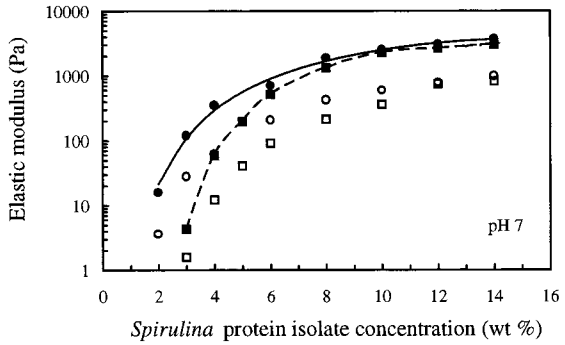

Figure 7. Concentration dependence of elastic moduli for *S. platensis* strain Pacifica protein isolate in 0.1 M Tris-HCl buffer, pH 7, at 90 °C (○) and 5 °C (●) and in 0.02 M $CaCl_2 \cdot 2H_2O$ in 0.1 M Tris-HCl buffer, pH 7, at 90 °C (□) and 5 °C (■). (1 Hz, 2% strain.)

proof that under some conditions protein gelation induced by heating does not need protein denaturation. The present results also confirm that under certain conditions protein−protein interactions and weak gel formation may precede denaturation in *Spirulina* protein isolate. Consequently, aggregation of condensed high-density and limited flexibility macroheterogeneous units of *Spirulina* protein molecules were present at the initial stages of the protein's network formation. The "gelation temperatures" discussed here should not be regarded as "gel points", signifying the appearance/disappearance of an infinite protein network. Our concern, here, is not to determine such gel points but rather to monitor those thermal shifts that follow changes in the rheological properties of the protein network formation.

Following the rheological characterization of the protein gels formed in buffer solutions, we now examine systems where salt is present. Upon addition of calcium chloride to the protein solutions, the samples exhibited similar viscoelastic behaviors during thermal heating and cooling; however, both elastic and loss moduli values of *Spirulina* gels were decreased (Figure 6a,b). Presumably this is a result of the lower solubility and perturbation of the electrostatic interactions of the protein in the presence of salt. The onset of the gelation temperature of the protein isolate in 0.02 M calcium chloride (which was defined when $G' > G''$ at a frequency of 1 Hz) decreases as the protein concentration was increased; for instance, for 2 and 4 wt % protein the gelation appeared at 63 and 54 °C, respectively. Overall, the onset of gelation of *Spirulina* protein in the presence of calcium was shifted to higher temperatures in accordance with the previous calorimetric data. As illustrated in Figure 6c, mechanical spectra at the end of the cooling confirm a gellike structure over all decades of frequency range ω (the elastic response, G', dominates over viscous flow, G'', with little frequency dependence in either moduli).

The development of the elastic modulus, G', as a function of *Spirulina* protein isolate concentration was monitored in the concentration range between 2 wt % (the minimum amount of protein required for the formation of a network with experimentally detectable gellike properties) and 14 wt % (the maximum protein concentration to ensure homogeneous samples). The values of G' extracted at 90 and 5 °C (the end of thermal heating and the end of the cooling run, respectively) were then used to describe the variation of network strength with protein concentration (Figure 7). Two

important pieces of information can be obtained from Figure 7: (i) *Spirulina* protein has the ability to develop strong network structures with high elastic modulus (i.e., >2.5 kPa at the end of the cooling for a concentration as low as 10 wt %). The minimum critical gelling concentration of heated and cooled *Spirulina* protein isolate preparations is of the order of ≈1.5 and ≈2.5 wt % in buffer solution and in buffer containing $CaCl_2$, respectively. These values are fairly low compared with the one obtained for the thermal gelation of other proteins; for instance, the minimum soy protein concentration for gelling is ∼8 wt % in distilled water at pH 7 (*40*−*42*). The low minimum protein concentration required for the gelation of *Spirulina* protein isolate may indicate that rather strong attractive forces between neighboring molecules occur in the *Spirulina* network structure. (ii) The elastic modulus can be represented by a power law behavior of $G' \sim c^x$, where c is the *Spirulina* protein concentration. The scaling exponents x, at both 90 and 5 °C (from the data of Figure 7), were a function of ionic strength and were as low as 2.7 in buffer solution and increased to 4.0 in $CaCl_2$. Although no clear distinction of a mechanism could be seen from these exponents, it is obvious that electrostatic interactions or protein solubility (in the presence of calcium chloride) affects the physical network formation of *Spirulina* protein gels. We will return to this point below. It is notable that the above scaling exponents of elastic moduli versus protein concentration are within the range of exponents observed for various protein gels, between 2 and 7 depending on experimental conditions (*43*, *44*).

Molecular Forces Involved in Thermal Association and Gelation of *Spirulina* Protein Gels. In what follows we report the *Spirulina* protein isolate functionality in different environments, such as different salts, reducing agents, polyols, urea, and sucrose, to elucidate the molecular forces responsible for the gelation mechanism. As illustrated in Figure 8a, there is a significant weakening of the network structure induced by sodium thiocyanate, whereas the gelation process remains unchanged. This is a phenomenon generally attributed to the weakening of hydrophobic interactions by the SCN^- ion (*18*, *45 46*). Because the involvement of hydrogen bonds or ionic links can been discounted, as they are weak or nonexistent at these temperatures, hydrophobic regions participate in intermolecular associations due to conformational changes of the protein under heating. The substantial increase in *Spirulina* proteins' hydrophobicity during heating and denaturation was as well confirmed by steady-state fluorescence measurements (Galatanu and Chronakis, unpublished data).

Propylene glycol is known to enhance protein−protein electrostatic interactions by decreasing the dielectric constant of water, to favor hydrogen bonding, and to hinder hydrophobic interactions (*24*). As shown in Figure 8a, the drastic decrease of the modulus on heating agrees with the diminishment of hydrophobic interactions that originally stabilize the network, whereas on cooling a further decrease of the network rigidity supports the involvement of hydrogen bonds in *Spirulina* protein gels. The G' values of 6 wt % protein with 20 and 50 wt % propylene glycol were 42.5 and 16.4 Pa at 5 °C, respectively. Therefore, hydrogen bonds alone are not sufficient to form and complement the network structure. Addition of an amphiphilic cosolute, such as ethylene glycol, was found to increase the modulus of the protein isolate during initial heating to 62 °C but caused a dramatic decrease and complete dissociation of the structure on further heating. These results suggest that increasing the solubilization of the initial aggregates, in the presence of ethylene glycol, inhibits any further structure formation and network reestablishment on further heating−cooling (data not shown).

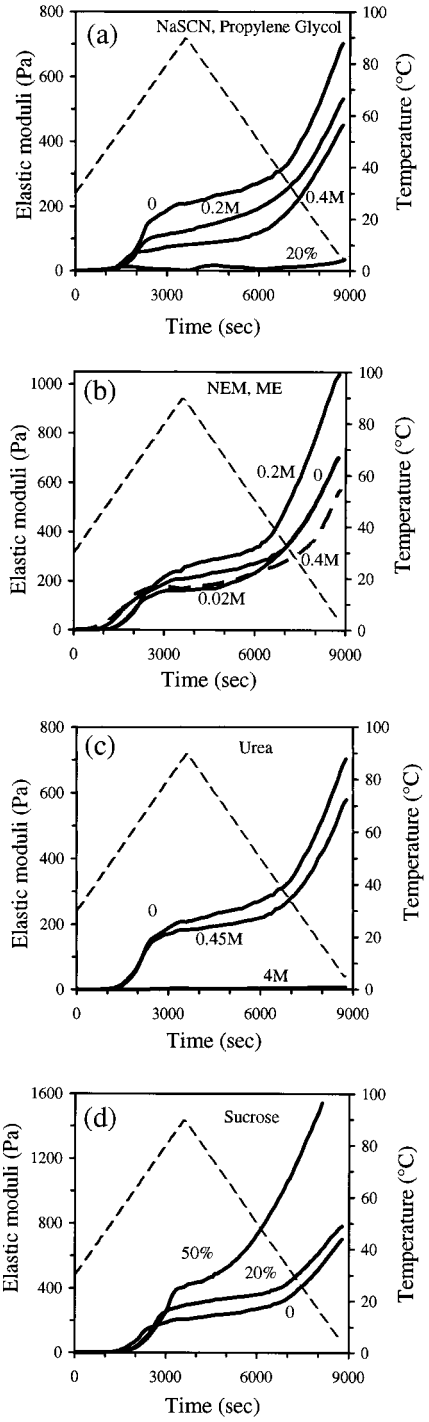

Figure 8. Cooling and heating profiles from dynamic oscillatory measurements of 6 wt % *S. platensis* strain Pacifica protein isolate in 0.1 M Tris-HCl buffer, pH 7, as a control sample (0) and in (a) 0.2 and 0.4 M NaSCN and 20% propylene glycol, (b) 0.02 and 0.2 M NEM and 0.4 M ME (- - -), and (c) 0.45 and 4 M urea; (d) 20 and 50% sucrose. All solutions were dissolved in 0.1 M Tris-HCl buffer, pH 7. Dotted line represents the temperature history. (1 Hz, 2% strain, 1 °C/min.)

The presence of 0.02 M *N*-ethylmaleimide (NEM) reduces the elastic modulus on heating (the gelation temperature remains unchanged at 43 °C), whereas on cooling the modulus of the protein gel was reinforced (Figure 8b). At 0.2 M NEM, the gelation temperature was at 38 °C and the modulus was further increased, probably due to the charge neutralization effects. Moreover, by the addition of 0.4 M 2-mercaptoethanol (ME) (which cleaves inter- and intramolecular disulfide bonds), formation and maintenance of a network structure were also created. These effects suggest that intermolecular sulfhydryl and disulfide bonds were not substantially involved in the establishment and structure of the network (*18*). Presumably, the disulfide reduction will also enhance the hydrophobic groups' exposure and their subsequent interaction during heating. Therefore, further breakage of most of the S−S bridges would not hinder the hydrophobic interactions on heating but would diminish the cross-linking density. Nevertheless, as observed in Figures 5 and 6, the network development was not increased considerably beyond ≈75 °C. This can be also due to an insufficient involvement of sulfhydryl−disulfide covalent interactions stiffening the performed aggregates and/or can be related to the maximum stability of the hydrophobic interactions involved. It has been estimated that generally the maximum intensity of hydrophobic interactions in protein−protein interactions is reached at different temperatures as a function of the amino acids involved (*47*).

Urea favors the gelation of some proteins, but, nevertheless, more frequently results in the destabilization of the gel structure. The urea effects are due to its interference with protein−protein hydrogen bonding or to the increase in the solubility of hydrophobic groups (*18*). The last action would result in weakening of the hydrophobic interactions on increasing the temperature and decreasing the elastic modulus on further cooling. Both types of action are shown in Figure 8c. Effective dissociation of the subunits requires treatment with high concentrations of urea (i.e., 4 M) or strong detergents such as 4 M ME or alkaline pH (>11.0).

Addition of sucrose as cosolvent enhances the overall structure of water, which indirectly strengthens hydrophobic interactions and network association (*24*). The magnitude of the stabilizing effect and the network rigidity increases with the amount of sucrose added (Figure 8d).

Furthermore, the *Spirulina* protein isolate was submitted to heating after calcium chloride enrichment. At concentrations of 0.004 M calcium chloride the protein gels showed higher elastic modulus during heating, either at neutral or at alkaline pH 9 (Figure 9). The decrease of elastic modulus in 0.02 M calcium chloride on heating can be attributed to charge neutralization effects and may reflect the involvement of electrostatic contributions in the network and their perturbation by added salt. On cooling, reduction of the network rigidity when the pH is 9 (independent of salt concentration) denotes that the increased solubility of hydrophobic groups diminishes the cross-linked network arrangement. Therefore, when the protein is more charged, it develops a weaker network, and this is also in accordance with the viscometric behavior of the protein at pH 9. It seems likely that salt affects protein−protein interactions due to electrostatic shielding and interference with hydrophobic interactions. At 5 °C the tan δ (G''/G') follows the order 0.02 M, pH 9 > 0.004 M, pH 9 > 0.004 M, pH 7 > 0.02 M, pH 7; hence, the cross-linked density was reduced at higher alkaline pH, whereas the stabilization and elasticity of the gels were favored by higher salt concentrations at neutral pH.

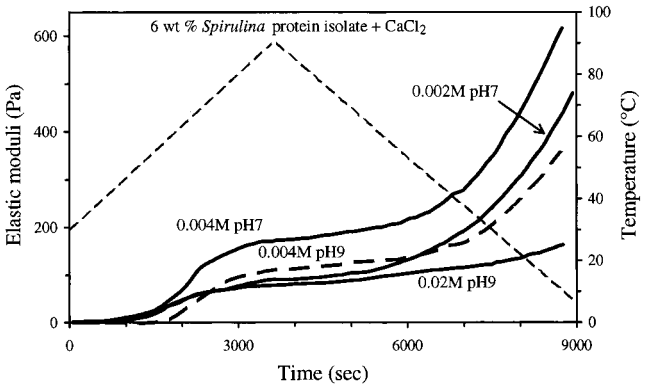

Figure 9. Cooling and heating profiles from dynamic oscillatory measurements of 6 wt % *S. platensis* strain Pacifica protein isolate in 0.004 and 0.02 M $CaCl_2 \cdot 2H_2O$ at pH 7 and 9 in 0.1 M Tris-HCl buffer. Dotted line represents the temperature history. (1 Hz, 2% strain, 1 °C/min.)

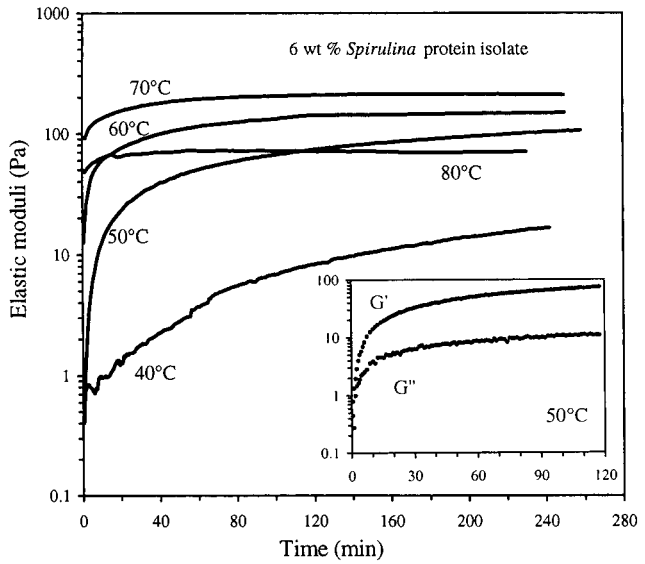

Figure 10. Effect of various temperatures (40−80 °C) on the elastic modulus (G') of 6 wt % *S. platensis* strain Pacifica protein isolate solutions. (Inset) Same data of the protein at 50 °C for the initial 120 min of heating. (1 Hz, 2% strain.)

Isothermal Gelation and Kinetics of *Spirulina* Protein Unfolding and Aggregation. A simple way to address some kinetic aspects of protein gelation is to examine the effect of heating temperature on the rate of development of elastic moduli. *Spirulina* protein isolate solutions (6 wt %) were heated at constant temperatures between 40 and 80 °C. As shown in Figure 10 the rate of heat-induced gelation and the gel strength exhibited a significant temperature and heating time dependence. The moduli were increased with time, as the temperature for curing was increased, attaining finally an asymptotic constant value. The highest moduli of the gels were observed at the isothermal temperature treatment of 70 °C, whereas at 80 °C the asymptotic value of the gel moduli was decreased. No data are shown for the 30 °C heat treatment because gel structures were not formed at this temperature even after 24 h of heating; thus, this protein concentration does not coagulate at temperatures below 40 °C.

The temperature dependence of the network development process monitored from these results could be attributed again to the temperature dependence of hydrophobic interactions. It is more likely that the main reason for the increase in G' is due to rearrangements of the protein hydrophobic groups. Note once more that formation of a protein coagulum can result from heating the *Spirulina* protein isolate below the denaturation temperature. A most likely explanation of the reduced elastic moduli of the gels formed at 80 °C is protein precipitation. Generally, at low protein concentrations, very rapid denaturation results in formation of large protein aggregates that either precipitate or form a grainy gel with large quantities of free water (syneresis) in the gel network. This could be also related to the ability of *Spirulina* protein gels to hold and bind water; however, in general, there is very little information available on the solvation and/or syneresis of gels formed from proteins. Additionally, the fast heating rate (80 °C) possibly may result in an increasingly random aggregation ("inhomogeneity") that causes irreversible changes in the internal protein structure and thus restricts the development of hydrophobic interactions.

Furthermore, on cooling to 5 °C the elastic moduli followed the same trends as obtained at the end of the heating process. G' increased in the order 70 > 60 > 50 > 80 > 40 °C of the temperature treatment (no results shown). The elastic character followed as well the same temperature dependence as the elastic modulus, either at the end of the heat treatment or at 5 °C. Therefore, the elasticity increases (lower tan δ values) in the order 70 > 60 > 80 > 50 > 40 °C of the temperature treatment. Possibly the increment in "permanent" elasticity may be attributed to an increasing involvement of intermolecular S−S bonds in the *Spirulina* network as a result of the additional thermal treatment.

CONCLUSIONS AND FURTHER CONSIDERATIONS

When we consider the denaturation and gelation properties of *Spirulina* algae protein, it is important to think that *Spirulina* protein isolate components are likely to be protein and/or protein−pigment complexes rather than individual protein molecules. Therefore, we can only discuss our data in qualitative terms.

As in other food proteins, we can regard the heat-induced denaturation and gelation of *Spirulina* protein as a two-step process: an unfolding step that is reversible and an aggregation step that mostly follows irreversible unfolding. Most possible is that when heat is applied to the protein solution, self-association and initial aggregation of exposed interior hydrophobic regions commence, giving the increase in the elastic moduli. Depending on the protein concentration, structures can be formed at temperatures below the heat denaturation temperature of *Spirulina* protein isolate. Beyond ≈60 °C protein denaturation reinforces the *Spirulina* protein gels and the initial interactions are strengthened further through a more extensive aggregation. The interactions of denatured molecules are irreversible. Higher temperatures (i.e., >100 °C) cause mainly irreversible chemical changes (such as the Maillard reaction in the case of sugar addition) with still unknown functional consequences. Finally, the gelation appears to have a final stage on cooling, and no changes were observed when the gels were cured at ambient temperatures.

Denaturation and gelation reached with or without salt (calcium) lead to quite different aggregation properties, possibly due to the effect of the salt on the protein solubility and the electrostatic interactions. At low ionic concentration, electrostatic interactions of charged protein molecules may be responsible for the higher elastic modulus than in the case without salt addition. However, the attractive interactions become less extensive and/or the solubility decreases at higher salt concentrations (i.e., 0.02 M $CaCl_2$), resulting in a decrease in the gel rigidity. When the protein is more charged (at alkaline pH), it develops a weaker network structure. Hydrophobic interactions have a significant role in *Spirulina* protein gelation and contribute predominantly in the molecular association, initial aggregation, and stability of the protein gels. The thermodynamic driving force for the hydrophobic association would be generated by the unstable state of exposed hydrophobic regions during the thermal treatment of the protein and the preference of these regions to associate with other such regions. Intermolecular disulfide bonds are not especially important in connecting the molecules but affect the physical properties of the gels, whereas hydrogen bonding complements the protein network structure on cooling. The low critical gelling concentration of *Spirulina* protein isolate, comparable with the critical concentration reported for the thermal association of other food proteins, is notable.

The heating process must be controlled so that it allows a high degree of coordination among *Spirulina* protein functional groups for network formation. Both time and temperature of heating strongly influence the rate of the network formation and the strength and the viscoelasticity of heat-induced *Spirulina* protein isolate gels. The rates of aggregation and gelation were more rapid as the temperature increased to 70 °C. Further increase of the heating rate (at 80 °C) diminished favorable protein−protein interactions and probably caused a more random coagulation and a low density of the ordered cross-links. This is in accordance with the lower elasticity (tan δ values) of the network structure formed at 80 °C. The strong temperature dependence of the network development process could be attributed again to the strong temperature dependence of the hydrophobic interactions. Although from the above kinetic study it is evident that the protein molecules associate and aggregate prior to the denaturation temperature, it is difficult to distinguish the temperature relationships between aggregation−gelation and denaturation involved in the structure formation of *Spirulina* protein isolate. Denaturation takes place progressively, passing through states with increased disorder. For the aggregation−gelation, the heating process must be controlled so that it allows a high degree of coordination and homogeneity among protein functional groups for a network formation.

In conclusion, it is evident that denaturation and gel formation in *Spirulina* protein isolate is a complex phenomenon. Better understanding of the stepwise denaturation and gelation requires studies of individual protein components in order to be resolved. Further work is need as well to establish the specific role of the protein−protein aggregates before and after the denaturation to the gelation properties. However, as far as applications are concerned where the rheological behavior of the system is the operating variable, rheological measurements such as the ones presented here

provide the most direct and relevant information. Nevertheless, on the basis of the analysis put forward above, we can generate questions and issues for other algae proteins as well. Knowledge of the structural−functional properties of algae proteins could create opportunities for tailoring them to the design of food products.

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