Heat-Induced Phenomena in Soy Protein Suspensions. Rheometric Data and Theoretical Interpretation

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Heat-induced aggregation of soy proteins in aqueous suspensions was studied through cone and plate rheometry for two different heating conditions. The rheometric data obtained covered the temperature range from 20 °C (stable colloidal suspension) to ∼90 °C (onset of network formation). Calorimetric data for the soy protein samples were also obtained to evaluate the degree of protein denaturation in the rheometric cell. Heat-induced transitions in soy globulins, such as dissociation, denaturation, and aggregation, were analyzed in relation to the rheological response of the suspension. The viscosity of the stable colloidal suspension satisfies the Cross model. A viscosity equation for the aggregating suspension was also derived by considering the fractal structure of the particle clusters and the Brownian aggregation mechanism. This equation is suitable to describe the experimental viscosity data.

Keywords: *Soy proteins; suspension rheology; aggregation kinetics; fractal dimension; viscosity model*

INTRODUCTION

The major components in soy protein isolates are globular. Most globular proteins suspended in water form aggregates and heat-set gels when the temperature is increased. This phenomenon is widely used in the food preparation and processing industry. In particular, soy proteins have a high nutritive value and an excellent capacity to improve the textural properties of foods (Giese, 1994).

Heat treatments of proteins weaken the bonds that maintain their secondary and tertiary molecular structure. As thermal denaturation occurs, the protein molecules expose to the solvent hydrophobic areas that are buried in the native conformation. This molecular change generates an aggregation process of the partially unfolded protein molecules as a consequence of the imbalance between attractive and repulsive forces of particles. At concentrations above a critical value, the aggregation process ends in the formation of a network. Therefore, any thermal history that changes the suspension from the stabilized colloidal state to the network formation gives rise to the following sequence of events: denaturation → aggregation → gelation (Schmidt, 1981; Clark and Ross-Murphy, 1987; Aguilera, 1995). Each of these transitions has associated with it different physicochemical processes. In fact, protein unfolding is accompanied by enthalpic changes that can be monitored by thermoanalytical techniques such as differential scanning calorimetry (DSC) (Scilingo and Añón, 1996; Relkin, 1994). On the other extreme, where the gradual development of viscoelasticity is observed, the gelation process is best characterized by measurement of the classical dynamic rheometric functions such as storage and dissipative moduli (Chronakis et al., 1995; Aguilera, 1995). The intermediate process, which involves colloidal aggregation, is probably the less studied phenomenon in the literature as far as globulins are involved. Nevertheless, we found several works recently published in which protein aggregation induced by heat was considered. For this purpose, different experimental techniques were used such as light and neutron scattering (Gimel et al., 1994; Renard et al., 1996), gel electrophoresis (Petruccelli and Añón, 1995), and ultracentrifugal sedimentation (Grinberg et al., 1992).

Within this context of analysis, the specific purpose of this work was, therefore, to get additional understanding of the aggregation process after protein denaturation and before the gel formation. The aggregation phenomenon was studied here through the classical shear rate rheometry. Experimental data covered the temperature range from the basic colloidal state until the onset of the network formation. A crucial point in our study was to propose an appropriate theoretical framework to relate the viscosity (macroscopic and measurable variable) with the size of the aggregates (microscopic variable), which changes with time and temperature. Additionally, a qualitative analysis of the colloidal stability of the soy protein suspension involving the forces between particles was proposed.

MATERIALS AND METHODS

Sample Preparation. Aqueous suspensions of native soy protein isolates were studied. The lyophilized soy protein samples were obtained with the experimental procedure described by Petruccelli and Añón (1995). The powder, which was highly soluble in water, consisted of >90% proteins and 8% moisture. The main protein fractions are 11S (340−375 kDa) and 7S (140−170 kDa) and constitute 52 and 35% of the isolated proteins, respectively. The other 13% are 2S and 15S

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fractions. Suspensions were prepared at ambient temperature at a concentration of 9.1% (w/w) protein powder in distilled water. After stirring for 30 min at 300 rpm, the suspension was stabilized overnight at 4 °C. Dispersed air was removed from the suspension before the rheometric test was carried out by applying a reduced pressure. All of the suspensions tested had a pH of 7.5. The filtration of the solution with a mesh ASTM No. 20 did not leave matter on the screen. At room temperature, we obtained a stable colloidal suspension of negatively charged (p$I \approx 4.5-5$; Hermansson, 1986) and hydrated particles in the semidilute range.

Sample Characterization. The averaged value of the hydrodynamic effective volume v_h of a protein molecule was calculated from the specific volumes of both solvent (v_1) and protein (v_2) by using the relation $v_h = (M_w/N_A)(v_2 + v_1\delta_1)$ (Tanford, 1961). In this equation, N_A is Avogadro's number, δ_1 measures the water bound to the proteins, and $M_w \approx 290$ kDa is the average molecular weight of proteins. Also, we adopted $v_2 = 0.75$ cm^3/g and $\delta_1 = 0.5$ g/g (Tanford, 1961; Fennema, 1977; Hiemenz, 1986). By assuming spherical particles, the averaged radius was estimated around $a \approx 5$ nm. Also, from the hydrodynamic volume obtained above, and considering the protein concentration in the suspension, the effective volume fraction of proteins obtained was $\phi = 0.107$. The mean surface-to-surface distance h_m between particles was obtained from $h_m = 2a[(\phi_{max}/\phi)^{1/3} - 1]$ (Russel et al., 1989). Adopting $\phi_{max} = 0.63$ resulted in $h_m \approx 1.6a$.

The characteristic Debye length of the diffuse double layer is $\kappa^{-1} = (\epsilon k_B T/2e^2 c N_A)^{1/2}$, where ϵ is the permittivity of the medium, k_B is the Boltzman constant, and e is the elementary electric charge (Hunter, 1992). Because there was no added salt to the suspension, c was evaluated as the concentration of counterions dissociated from the globulins. Thus, $c = zc_p$, where z is the mean number of net charges on protein surfaces and c_p is the protein concentration. Assuming that for soy proteins suspended in water at pH 7.5 the number z was ~25, we obtained $\kappa^{-1} \approx 3.3$ nm; that is, $\kappa a \approx 1.5$ at 20 °C. In addition, the mean surface potential of globular protein molecules was estimated as $\iota_0 = ze/4\pi\epsilon a(1 + \kappa a)$ (Tanford, 1961). From the values of z and κa calculated above, the result was $\iota_0 \approx 35.6$ mV at 20 °C.

Rheometric Tests. A closed and sealed cone and plate cell in a Brookfield programmable rheometer of low viscosity range was used. The cell characteristics are as follows: cone radius $R_c = 24$ mm; cone angle $\alpha_0 = 1.565°$; sample volume $V = 1$ mL. This rheometer includes software (Brookfield Rheocalc) that permits data analysis and systematic recordings of torque M, rotational speed ω, time t, and temperature T. The shear stress τ and the shear rate $\dot{\gamma}$ in this cell are $\tau = 3M/2\pi R_c^3$ and $\dot{\gamma} = \omega/\alpha_0$, respectively (Walters, 1975). Consequently, the viscosity is $\eta = \tau/\dot{\gamma}$, which depends on shear rate, temperature, and time. In this work, the viscosity of the suspension samples was measured for the rheometric tests described next.

Test A. The viscosity was measured as function of temperature T in the range of 20−90 °C for a fixed value of shear rate. The cell temperature T_h increased linearly with the rate of heating $\beta = 2.3$ °C/min. Therefore, the thermal and mechanical histories applied to the sample were $t < 0$, $T_h = T_0$, and $\dot{\gamma} = 0$ and $t \geq 0$, $T_h = T_0 + \beta t$, and $\dot{\gamma} = \dot{\gamma}_0$, where subscript 0 indicates that the variable was evaluated at the initial time $t = 0$. Thus, T_0 was the initial temperature of the sample. The evolution of the sample temperature T could be evaluated through a simple energy balance (Berli and Deiber, 1997). It is thus found that $T \approx T_h$ for the rheometric cell and the rate of heating used. In this test, the interpretation of rheometric data was, of course, simpler than for the case where T might differ from T_h. Measurements at high temperatures ($T > 70$ °C) were replicated three times, obtaining highly reproducible data.

Test B. The viscosity was measured as function of time while the temperature and the shear rate were kept constant. This test covered the temperature range from 20 to 90 °C. The thermal and mechanical histories applied to the sample were $t < 0$, $T = T_0$, and $\dot{\gamma} = 0$ and $t \geq 0$, $T = T_h$, and $\dot{\gamma} = \dot{\gamma}_0$. Thus, because the rheometric cell was initially at ambient temperature T_0, the sudden imposition of temperature T_h to the sample was obtained by allowing the circulation of the bath fluid from $t = 0$. Under these operating conditions the average temperature of the sample T increased toward the cell temperature T_h according to $(T - T_h)/(T_h - T_o) = \exp(-t/t_c)$. This equation was obtained through the energy balance in the rheometric cell. It was found that for the type of suspensions studied in this work $t_c \approx 10$ s (Berli and Deiber, 1997).

Table 1. Parameters for the Kinetics of Denaturation of Soy Protein Fractions

parameter	7S	11S
p_i	3.5	2
$k^0_{d,i}$ (s^{-1})	2.9×10^{100}	1.0×10^{53}
$\Delta E_{d,i}$ (kJ/mol)	683.2	381.4

Calorimetric Test. DSC thermal analysis was performed in a Rheometric Scientific differential scanning calorimeter of Polymer Laboratories, driven by Plus V5.41 software. Calibration was done with indium and stearic acid (ASTM Norms E 698/79 and E 474/80). Hermetically sealed aluminum pans were prepared to contain 15 mg of soybean protein isolate suspended in distilled water (20% w/v). The samples were scanned at 10 °C/min from 30 to 130 °C. An empty double capsule was used as reference. After each DSC analysis, the pans were punctured, and the dry matter weight was determined by drying at 105 °C overnight. The enthalpy of denaturation ΔH and the peak temperature T_p were also obtained by using the Plus V5.41 software.

RESULTS AND DISCUSSION

Calorimetry. Thermal denaturation thermograms of our sample exhibited the two characteristic peaks corresponding to endothermic transitions of the 7S and 11S fractions (Scilingo and Añón, 1996). The peak temperatures T_p were 78.9 and 94.2 °C for 7S and 11S, respectively. To interpret later the experimental data of the suspension viscosity, the degrees of denaturation of proteins composing the sample are required, according to the thermal histories of the tests. For these purposes, the following denaturation kinetics were considered (Borchardt and Daniels, 1957):

$$\frac{d\alpha_i}{dt} = k^0_{d,i} \exp\left(-\frac{\Delta E_{d,i}}{RT}\right)(1 - a_i)^{p_i} \qquad (1)$$

In eq 1, α_i is the degree of denaturation of the 7S ($i = 1$) and 11S ($i = 2$) protein fractions, so that $0 \leq \alpha_i \leq 1$, and p_i is the reaction order in relation to the undenatured protein fraction $(1 - \alpha_i)$. Also, $k^0_{d,i}$ is the Arrhenius frequency factor, $\Delta E_{d,i}$ is the activation energy, and R is the gas law constant. The kinetic parameters in eq 1 were obtained from DSC data with the method suggested by Borchardt and Daniels (1957). The results of the analysis carried out here are reported in Table 1. It is interesting to point out that the reaction orders p_i obtained in this work have the same value as those reported by Scilingo and Añón (1996), who analyzed the 7S and 11S globulin-rich fractions separately. Once the parameters p_i, $k^0_{d,i}$, and $\Delta E_{d,i}$ of each protein fraction are available, the degree of denaturation can be calculated for any rate of heating β with $d\alpha_i/dt = \beta\, d\alpha_i/dT$. This procedure is valid when the enthalpy of denaturation ΔH does not change appreciably with the rate of heating. This was experimentally proved for our soy protein suspension. In fact, we obtained $\Delta H = 16.3 \pm 1.7$ J/g for any heating rate between 5 and 25 °C/min.

Figure 1 presents the rate of denaturation of each protein as function of temperature by using data from Table 1. In this figure, solid lines refer to the rate of

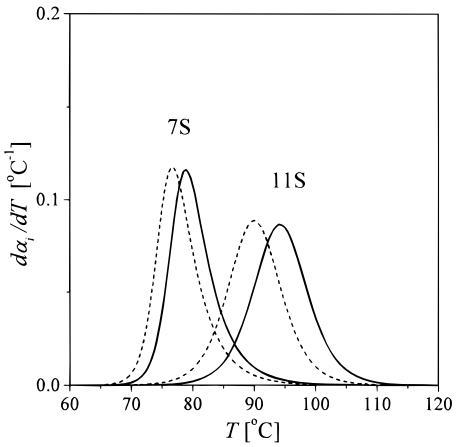

Figure 1. Rate of denaturation as a function of temperature for 7S and 11S soy protein fractions at different heating rates: solid lines, $\beta = 10$ °C/min (DSC test); dashed lines, $\beta = 2.3$ °C/min (rheometric test).

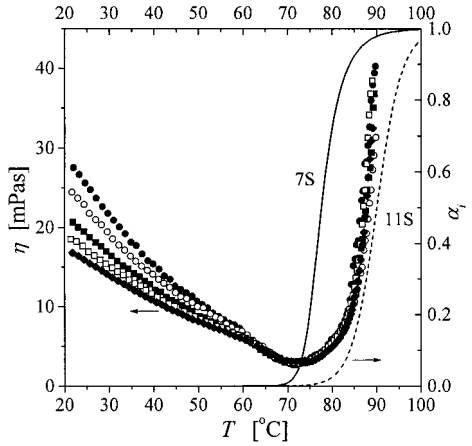

Figure 2. Experimental viscosity η as function of temperature T for the soy protein suspension. Symbols are the values obtained with the rheometric test A at different shear rates: (●) 114, (○) 190, (■) 304, (□) 456, and (◆) 646 s^{-1}. Solid and dashed lines represent the degrees of denaturation α_1 and α_2 of 7S and 11S protein fractions in the rheometric cell, respectively. They were calculated with eq 1 and parameters reported in Table 1 for the heating rate $\beta = 2.3$ °C/min.

heating utilized in the DSC test. Dashed lines refer to $\beta = 2.3$ °C/min and are of interest in the interpretation of the rheometric test A. One can observe that with decreasing β, temperature T_p decreases around 3 °C for the 7S fraction and 4 °C for the 11S fraction. These thermal shifts are compatible with previous experimental results (Scilingo and Añón, 1996). In our DSC experiments, neither the peak temperature T_p nor the enthalpy ΔH varied significantly between 10 and 20% of protein concentration. Therefore, we use the kinetic parameters obtained from DSC data as a good approximation to predict the protein denaturation degrees in the rheometric tests, which were carried out at 9.1% of protein concentration.

Rheometry. Figure 2 shows the thermal evolution of the suspension viscosity in the temperature range of 20−90 °C. The experimental data shown were obtained with the rheometric test A. There are specific behaviors of the protein suspension in this test showing clearly that the experimental data can be analyzed by considering three thermal zones.

Basic Colloidal State ($T < 55$ °C). In this temperature range, the suspension is shear-thinning. This rheological behavior of the soy protein suspension in shear flow is well described by the Cross model:

$$\eta = \eta_\infty + \frac{\eta_0 - \eta_\infty}{1 + (\lambda\dot{\gamma})^m} \qquad (2)$$

In eq 2, η_0 and η_∞ are the asymptotic viscosity values, corresponding to $\dot{\gamma} \rightarrow 0$ and $\dot{\gamma} \rightarrow \infty$, respectively. Also, λ is a characteristic time and $m > 0$ (Cross, 1965). The values of the rheological parameters η_0, η_∞, and λ of eq 2 are presented in Table 2, which were evaluated with the method proposed by Cross and with experimental data of test B at different shear rates, for $T < 55$ °C. In Table 2, $m = 0.45$ and r is the correlation coefficient. Figure 2 shows that degree of shear-thinning decreases when temperature increases, and for $T > 60$ °C the suspension is almost Newtonian. These results are also expected theoretically. In fact, at high temperatures the Brownian motion predominates over the convective motion of the flow, resulting in an equilibrium structure with the limit viscosity $\eta \rightarrow \eta_0$ because $\lambda\dot{\gamma} \rightarrow 0$ (see the thermal evolution of λ in Table 2). For the stabilized suspension of charged particles, η_0 can be described as composed of two terms (Ogawa et al., 1997). One term is the well-known Krieger−Dougherty equation (Krieger, 1972), and the second term is the contribution of the electrical forces to the suspension viscosity (second electroviscous effect) through an activation energy of the order of $\epsilon\psi_0^2 a$. In fact, the electrostatic repulsion yields viscosity values ~20 times greater than those expected for an equivalent suspension of neutral particles, which in general has a viscosity around twice that of the solvent, η_s. This effect is also a consequence of the hydration repulsive forces, which are relevant to stabilize the basic colloidal state, as will be discussed later. The viscosity decreases with temperature due to the decrease of the medium viscosity and, mainly, to a significant decrease of the pair interaction energy of colloidal forces in relation of the Brownian thermal energy.

Table 2. Rheological Parameters of the Cross Model for the Soy Protein Suspension at Low Temperatures, Obtained from Equation 2, where $m = 0.45$

T (°C)	η_∞ (mPas)	η_0 (mPas)	λ (ms)	r
20	1.39	62.40	14.0	0.9998
30	1.11	38.14	5.95	0.9998
40	0.91	21.41	2.19	0.9980
50	0.76	13.74	1.09	0.9970

Intermediate Zone (55 °C $< T < T_{min}$). This zone starts at the inflection point of $\eta(T)$, approximately at 55 °C, and ends around $T_{min} \approx 72$ °C, where the viscosity presents a minimum value, as shown in Figure 2. The more rapid decrease of the viscosity with temperature in this zone is associated with the disruption of the quaternary structure of globulins by dissociation of the subunits. In fact, enough information exists in the literature (German et al., 1982; Hermansson, 1986; Utsumi et al., 1987) indicating that glycinin (11S fraction) and β-conglycinin (the main component of the 7S fraction) have quaternary structures which dissociate into subunits under specific conditions of temperature, pH, and ionic strength. Glycinin is an oligomeric protein that consists of six subunits, and β-conglycinin is a trimeric protein. Both of them can be fully dissociated by heating (German et al., 1982; Hermansson, 1986; Petruccelli and Añón, 1995). In this sense, from the rheological point of view one can point out the follow-

ing: (a) The dissociation may yield a particulate system of more uniform sizes, that is, the polydispersity decrease. This hypothesis is based on the fact that in the dissociation process the average radii of glycinin ($a \approx 6$ nm, ~360 kDa) and conglycinin ($a \approx 4.5$ nm, ~156 kDa) evolve approximately to $a \approx 3.3$ nm (6 units of ~60 kDa) and $a \approx 3.1$ nm (3 units of ~52 kDa) respectively, at the complete dissociation [see also Utsumi et al. (1987)]. Nevertheless, this effect decreases the maximum volume fraction ϕ_{max} and hence the viscosity increases, indicating that it cannot be responsible for the phenomenon under analysis. (b) Occluded solvent in the quaternary structure may be released with the dissociation. However, the decrease in the effective volume fraction required to explain the observed decrease in viscosity cannot be attained by decreasing the bulk hydration of particles (δ_1). (c) The decrease in particle size reduces the average distance between particles, for a fixed value of ϕ. Nevertheless, this effect increases the viscosity (rather than decreases it) due to the higher electrostatic repulsion, which is a consequence of the overlapping of the electric double layers (Ogawa et al., 1997). As far as the above analysis is considered, it seems there is not yet a definite explanation of the phenomenon observed in this zone. Nevertheless, an interesting hypothesis emerges from the consideration of the surface charge conservation of protein particles. Thus, each dissociated globulin has to maintain a fraction of the charge in the original quaternary structure. A simple calculation indicates that, when globulins dissociate, the scale of the repulsive interaction energy between particles $\epsilon\psi_0^2 a$ (Russel et al., 1989) diminishes around 4 times for both 7S and 11S globulins, which results in a lower value in the suspension viscosity. Additionally, the decrease of particle radius a reduces the hydration force (Israelachvili, 1995). Therefore, we can assume that the rheological response in the intermediate zone is a consequence of an appreciable decrease of electrostatic and hydration repulsive forces due to a smaller averaged particle diameter of the proteins in suspension.

Aggregation Zone ($T > T_{min}$). Near above 72 °C, the suspension viscosity increases with temperature, and this phenomenon coincides with the onset of the denaturation of globulins. The stability of the suspension breaks down, and the hydrophobic interaction is one of the controlling mechanisms that emerges as a consequence of the partial denaturation of the globulins. The denaturation process also reduces the superficial hydration layer of globulins (Fennema, 1977), which yields a substantial reduction of the repulsive hydration force (van Oss, 1997). Therefore, one expects the aggregation of the suspending particles to form clusters of bigger sizes. In Figure 2, it is observed that the processes of denaturation and aggregation (this last associated to the increase of viscosity) are in a clear sequence. Thus, the fraction 7S denatures significantly before a substantial increase in viscosity is observed. The denaturation of the 11S fraction occurs at higher temperatures, where the suspension viscosity diverges. The rheological response above 72 °C is modeled and analyzed quantitatively in the next section.

In a general context of analysis of the three zones described above, the basic colloidal state approaches the behavior of a Newtonian fluid as the temperature is increased. Thus, all of the curves in Figure 2 converge to a common asymptote. This behavior is clearly evident

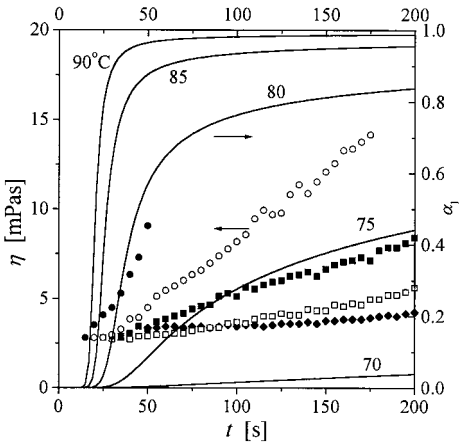

Figure 3. Experimental viscosity η as function of time t for the soy protein suspension. Symbols are the values obtained with the rheometric test B (shear rate 304 s^{-1}) at different temperatures: (◆) 70, (□) 75, (■) 80, (○) 85, and (●) 90 °C. Lines represent the degree of denaturation of 7S protein fraction in the rheometric cell. They were calculated with eq 1 and parameters reported in Table 1 according to the thermal evolution of the sample at each temperature.

in the intermediate zone, where the dissociation of proteins generates a suspension of small and low charged particles. In the last zone, where the aggregation is important, the effect of the shear rate is not significant on the value of the viscosity and the changes in the microstructure are irreversible. However, although at the intermediate zone the suspension can be considered a Newtonian fluid, in the third zone, this result cannot be assured anymore because an important buildup of microstructure occurs suddenly. In this sense, it must be pointed out that in this zone rheometric data are obtained during the initial step of the aggregation process, where it is possible to maintain a shear rate flow. Later the aggregates interconnect themselves to form a network, giving place to the onset of the gelation process.

Specifically for the aggregation process, Figure 3 presents experimental data obtained with test B. Symbols show the measured suspension viscosity as function of time t for different values of heating temperature T_h. These data were useful to evaluate the viscosity equation proposed in this work for the aggregating suspension. In addition, solid lines in Figure 3 show the degree of denaturation of the 7S fraction. For temperatures >80 °C, the 7S fraction denatures almost fully in ~30 s. For $T_h = 70$ °C, the degree of denaturation is very low after 2 min. Nevertheless, a smooth increment of viscosity as function of time occurs at this temperature. In this sense, it should be pointed out that the high sensitivity of the rheometric technique enables one to detect low fractions of aggregates and low rates of aggregation, as well as the importance of quantifying the denaturation process, which allowed us to compare the degrees of denaturation with the experimental values of viscosity.

Mechanism of Aggregation. The problem of aggregation in the flowing suspension is a central subject in this work. In colloid science, two limit cases are usually considered: perikinetic aggregation, where the particle encounters are due to Brownian motion, and orthokinetic aggregation, where the collisions are caused by the gradient velocity field. The relative importance of each mechanism can be measured by evaluating the number density flux of particles per unit area J (Russel et al., 1989). The result is $J_{orth}/J_{peri} = 4a^3\eta_s\dot{\gamma}/k_BT = 4Pe$,

where *Pe* is the Peclet number defined as the ratio of the Brownian time scale to shear rate time scale. For the aqueous soy protein suspensions of this work, where the averaged particle radius was $a \approx 5$ nm and the maximum value of shear rate used in tests A and B was $\dot{\gamma} = 646\ s^{-1}$, we obtained $Pe < 10^{-5}$. This result indicated that in these soy globulin suspensions, the principal collision mechanism was due to Brownian aggregation. In fact, it is known that orthokinetic aggregation dominates for particles of the order of micrometer size and larger. Nevertheless, the combination of Brownian motion and a velocity gradient should be considered in the rheometric cell. Following the analysis of Russel et al. (1989), for low Peclet numbers, the degree of enhancement in the aggregation rate due to shear implies that J has to be multiplied by the factor $(1 + 0.257Pe^{1/2}/W)$. Here, W is the stability ratio

$$W = 2a \int_{2a}^{\infty} \frac{\exp[U_T(r)/k_B T]}{G(r)r^2}\, dr \qquad (3)$$

where $U_T(r)$ is the total interaction energy between the particles and $G(r)$ is the hydrodynamic correction factor (Russel et al., 1989). In the most critical situation, where $W \approx 1$, and for $Pe < 10^{-5}$, a simple calculation indicates that shear increases the aggregation rate <1% over the value corresponding to that of the Brownian mechanism. These theoretical predictions are in agreement with the experimental results of our work, where $\dot{\gamma}$ does not alter the aggregation process, as is observed in Figure 2.

There are two main factors producing disaggregation in a colloidal suspension under shear flow: Brownian motion and mechanical stress due to viscous forces. These possibilities were also taken into account in our analysis. Because the Peclet number for aggregates, Pe_a, is very low at the first steps of the aggregation ($Pe_a = R_a^3 \eta_s \dot{\gamma}/k_B T < 10^{-3}$, where R_a is the average aggregate radius), the shear rate does not significantly affect the growth of clusters (Potanin, 1991). Thus, if breakup of aggregates occurs, it is due to Brownian motion mainly. Therefore, it is necessary to analyze the importance of the thermal energy $k_B T$ as a factor of disaggregation. A reasonable approximation can be obtained by balancing the interparticle bond energy in the aggregates U_b against $k_B T$ (Russel at al., 1989; Potanin, 1991). The bond energy U_b may be estimated by assuming that, in the first steps of the heat-induced soy protein aggregation, the principal force involved is the hydrophobic one. For instance, for a pair of protein molecules with hydrophobic surface domains of ~4 nm^2, the attractive energy at contact is approximately $-25k_B T$ (van Oss, 1997). Then, because $|U_b| \gg k_B T$, it can be assumed that the soy protein aggregates are not substantially destroyed by Brownian motion either.

Therefore, in this context of analysis, we describe the aggregation process of denatured soy globulins according to the Brownian aggregation theory, by which the rate of disappearance of primary particles in the system is

$$-dn/dt = k_a n^2 \qquad (4)$$

n being the number density of particles (kinematic units) in the suspension at time t. In the classical Smoluchowski theory, the rate constant is $k_a = 4k_B T/3\eta_s = k_S$, the well-known Smoluchowski rate constant. This regime is designated rapid flocculation or diffusion-limited aggregation (DLA). When the presence of an energy barrier between particles slows down the collision frequency, the rate constant becomes $k_a = k_S/W$, resulting in slow flocculation or reaction-limited aggregation (RLA). It is important to mention here that the validity of eq 4 implies certain hypotheses already discussed precisely by Russel et al. (1989). They are (a) cluster−cluster aggregation of equal sizes predominates and (b) the stability ratio does not depend on cluster sizes. Therefore, we adopted this procedure and, hence, used eq 4 throughout this work. For later use, we also designate $t_{1/2} = 1/k_a n_0 = W/k_S n_0$ the half-life time for the number of particles in the suspension, at which n is reduced to half of its initial value n_0.

Structure of Aggregates. Recent experimental and theoretical results found in the literature show that the aggregation of colloidal particles leads to the formation of fractal structures, that is, highly branched aggregates. In fractal aggregates, the averaged number N of particles in a cluster, also designated degree of aggregation here, is related to its average radius R_a according to (Russel et al., 1989)

$$N = (R_a/a)^f \qquad (5)$$

The fractal dimension $f < 3$ is a measure of the internal density of the aggregates, and it depends on the rate of aggregation. In general, DLA yields structures with $f = 1.75$, when cluster−cluster aggregation predominates, whereas RLA gives $f = 2-2.2$ (Russel et al., 1989). In this theoretical context, one can derive an expression for the volume fraction of aggregates ϕ_a as a function of the number of particles per aggregate N. Thus

$$\phi_a = \phi N^{(3-f)/f} \qquad (6)$$

These concepts are used next to develop a viscosity equation for the aggregating soy protein suspension. In particular, the aggregates formed upon heat-induced denaturation of globular proteins have a fractal dimension that varies between 1.75 and 2.05 (Gimel et al., 1994; Renard et al., 1996; Aymard et al., 1996). Hagiwara et al. (1997) reported higher values of f (between 2.00 and 2.82) from the analysis of heat-induced protein gels.

Viscosity of the Aggregating Suspension. When $T > T_{min}$, at which denaturation of globulins is important and clusters of particles are formed, the viscosity equations mentioned above do not apply in a simple physical context. To study this situation, we propose to develop the viscosity equation for the aggregating soy protein suspension by using the modified Krieger−Dougherty equation [see also Quemada (1977)]. Therefore, assuming that the discrete phase is composed predominantly of aggregates, we substitute ϕ_a for ϕ and the viscosity of the medium η_m that fills the space between the aggregates for η_s, as suggested by several authors (Potanin, 1991; Hunter, 1992; Lapasin et al., 1996). Therefore

$$\frac{\eta}{\eta_m} = \left[1 - \frac{\phi}{\phi_{max}} N^{(3-f)/f}\right]^{-2} \qquad (7)$$

This equation relates the number of particles per aggregate with the suspension viscosity. The next step in our derivation consists of including in eq 7 the

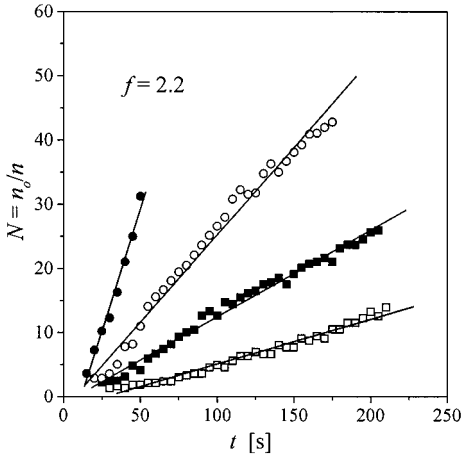

Figure 4. Number of particles per aggregate N as function of time t for the aggregating soy protein suspension. Symbols are the values obtained from viscosity data by using eq 9, where $f = 2.2$, at different temperatures: (□) 75, (■) 80, (○) 85, and (●) 90 °C. The straight lines refer to the prediction of the perikinetic aggregation theory at each temperature (eq 8).

appropriate kinetic expression for the degree of aggregation N. In this sense, first we use eq 4, which can be readily solved for a constant temperature. Then, by using the identity $N = n_0/n$, the following expression is obtained:

$$N = 1 + \bar{k}_a(t - t_0) \tag{8}$$

In eq 8 $\bar{k}_a = n_0 k_a$ and t_0 is the initial time at which $N \approx 1$. Time t_0 has to be evaluated through the fitting procedure described next because the steady-state temperature of test B was reached after ~10 s. Also, by rearranging eq 7 one can obtain N

$$N = \left\{\frac{\phi_{max}}{\phi}\left[1 - \left(\frac{\eta_m}{\eta}\right)^{1/2}\right]\right\}^{f/(3-f)} \tag{9}$$

This equation allows us to measure the degree of aggregation in the suspension from viscosity data at a given time and temperature. For this purpose, the fractal dimension is important, because it involves the principal features of the aggregation process. Here, we propose to estimate the value of f from rheometric data of our soy protein suspension.

To compare the model with experimental data, we adopted $\phi_{max} = 0.63$, which corresponds to the regime of low Peclet numbers (Russel et al., 1989). The value of the medium viscosity η_m was obtained from suspension viscosity data at the temperature T_{min}, where $N \approx 1$. Experimental data from test A indicated that $\eta_m \approx 4.73\eta_s$. Then, the number of adjustable parameters in the viscosity model reduces to f, $\bar{k}_a$, and t_0. To quantify these parameters, the following procedure was used. The value of f required in eq 9 to fit eq 8 to calculated data was found. In this sense, the lineal regression of N as function of t (given by eq 9) was carried out and is shown in Figure 4. Sets of data at different temperatures obtained from rheometric test B were used. The best correlation of data with eqs 8 and 9 was obtained for $f = 2.2$ (correlation coefficient $r > 0.988$). Simultaneously, $\bar{k}_a$ (slope of the straight lines in Figure 4) and t_0 values at each temperature were obtained and are reported in Table 3.

The fractal dimension obtained here has a value that corresponds to RLA (Russel et al., 1989). In the next section we conclude that RLA is the predominant mechanism in the heat-induced aggregation of the soy protein suspensions studied here. In addition, f seems to be constant in the temperature range of our experiment. This is in accord with the results obtained previously by Gimel et al. (1994) for globular proteins. The value of f obtained with our soy globulins is slightly higher than those reported for β-lactoglobulin (Gimel et al., 1994; Renard et al., 1996; Aymard et al., 1996). This may be attributable to several factors, such as some degree of cluster−particle aggregation, which leads to more compact aggregates (Russel et al., 1989). In addition, the soy protein suspensions studied here are polydisperse and the aggregation may form denser structures. Nevertheless, it should also be considered that different experimental techniques were used in each case, apart from the globulins being chemically and structurally different.

Table 3. Aggregation Rate Constant at Different Temperatures, Obtained from Rheometric Data by Using Equations 8 and 9 with $f = 2.2$

T (°C)	$\bar{k}_a$ (s^{-1})	t_0 (s)	r
75	0.070	42	0.9883
80	0.135	15	0.9937
85	0.272	12	0.9922
90	0.760	12	0.9906

Once the fractal dimension f in our system was obtained, the viscosity model could be applied to rheometric data of Figure 2, in the aggregation temperature zone ($T > T_{min}$). In this sense, one can also calculate $\bar{k}_a$ from rheometric data with a different thermal history (test A) and compare it with the values already available in Table 3. In fact, N versus T was obtained from experimental data of η versus T by using eq 9. According to the thermal evolution of the sample during the rheometric test A, eq 4 is rewritten as follows

$$-\beta(dn/dT) = k_a n^2 \tag{10}$$

By using $N = n_0/n$ and assuming that k_a obeys the Arrhenius function (Hiemenz, 1986), eq 10 can be converted to the following expression

$$\ln\left(\frac{dN}{dT}\right) = \ln\left(\frac{\bar{k}_a^0}{\beta}\right) - \frac{\Delta E_a}{RT} \tag{11}$$

This equation allowed us to calculate parameters $\bar{k}_a^0 = n_0 k_a^0$ and ΔE_a by applying the linear regression method in an Arrhenius plot. The value obtained for the activation energy ΔE_a was 171.6 ± 8 kJ/mol. The corresponding value obtained from the rheometric test B (Table 3) was 165.0 ± 14 kJ/mol. It is observed that both predictions are quite coincident, despite the error that is inherent in the method of calculation. Figure 5 shows the prediction of the model in the aggregation temperature zone (eqs 7 and 10). A good agreement between experimental data and the theoretical $\eta(T)$ curve is observed. Therefore, it is appropriate to mention here that the viscosity model proposed for the aggregating soy protein suspension represents well the experimental data obtained under the two different heating conditions used here.

Suspension Stability and Protein Denaturation. At the pH of the suspending medium, the suspended globulins are electrically charged. Additionally, protein surfaces are strongly hydrophilic and the superficial

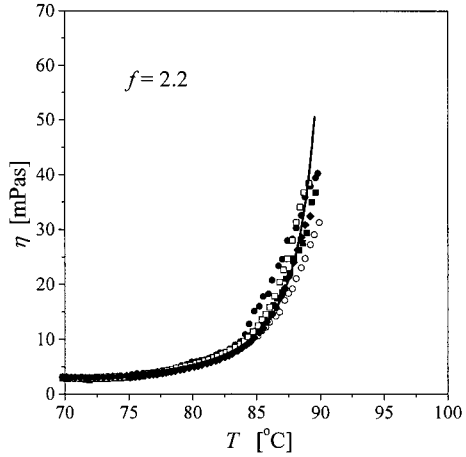

Figure 5. Viscosity η as function of temperature T for the aggregating soy protein suspension. Symbols are the experimental data at different shear rates: (●) 114, (○) 190, (■) 304, (□) 456, and (◆) 646 s^{-1}. The solid line is the prediction of the viscosity model (eqs 7 and 10) with $f = 2.2$.

hydration generates a significant repulsive force between pairs of particles (Hunter, 1992; van Oss, 1997). Nevertheless, when the thermally induced unfolding of molecular chains occur, the proteins expose nonpolar residues that confer a hydrophobic character to the particle surface. The immediate results are a decrease in hydrophilic repulsion due to the loss of superficial hydration of globulins [see Fennema (1977)] and the onset of attractive hydrophobic energy. In this theoretical context, and assuming spherical particles separated by a surface-to-surface distance h, the protein−protein interaction energy U_T in the suspension has the following components:

$$U_R = \frac{4\pi\epsilon\psi_0^2 a^2}{h + 2a}\exp(-\kappa h) \quad (12)$$

$$U_A = -\frac{A}{6}\left\{\frac{2a^2}{h^2 + 4ah} + \frac{2a^2}{h^2 + 4ah + 4a^2} + \ln\left(\frac{h^2 + 4ah}{h^2 + 4ah + 4a^2}\right)\right\} \quad (13)$$

$$U_S = \Delta G_{pwp}\pi a l \exp(-h/l) \quad (14)$$

Equation 12 represents the energy of repulsion due to the interaction of the electrical double layers surrounding the particles. Equation 13 represents the attractive van der Waals interaction energy, where A is the Hamacker constant. U_R and U_A are the DLVO (Derjaguin−Landau−Verwey−Overbeek) pair potential components (Russel et al., 1989; Hunter, 1992). Here, we adopted the value $A = 10k_BT$, reported for protein−water−protein systems (Coen et al., 1995). Equation 14 estimates the interaction energies due to the solvent, also designated non-DLVO interactions (Israelachvili, 1995). Here, U_S was obtained from data involving surface−surface interactions reported in the literature (Israelachvili, 1995; van Oss, 1997) and using the Derjaguin approximation (Hunter, 1992). The free energy ΔG_{pwp} measures the absolute degree of hydrophilicity or hydrophobicity between two proteins in water, and $l \approx 1$ nm is the exponential decay length (van Oss, 1997; Israelachvili and Wennerström, 1996). Although these equations and parameter values are not definitive for soy proteins in water, they are reasonable approximations for our purposes.

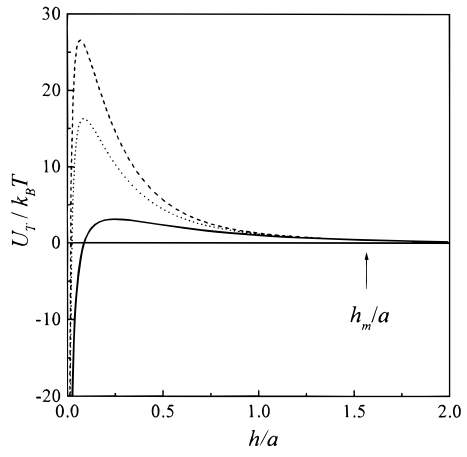

Figure 6. Pair interaction potential energies U_T normalized with the Brownian thermal energy k_BT as function of the dimensionless surface-to-surface distance h/a for soy globulin particles, estimated according to eqs 12−14. The solid line represents $U_R + U_A$ at 20 °C (DLVO theory). The dashed and dotted lines refer to $U_R + U_A + U_S$ at 20 and 80 °C, respectively. Data for numerical calculations are as follows: $z = 25$, $a = 5$ nm, $A = 10k_BT$, $l = 1$ nm, $\Delta G_{pwp} = 10.5$ mJ/m^2 at 20 °C, and $\Delta G_{pwp} = 7.9$ mJ/m^2 at 80 °C.

The approximate number of particles per cubic meter in our suspension was 2×10^{23}. If one assumes that the half-life time is at least 1 day for the stabilized suspension, the stability ratio W takes a value of $\sim 10^{11}$ at 20 °C. Nevertheless, from eq 3 it was found that the DLVO forces give $W \approx 10^1$ at 20 °C. Because we obtain a soy protein suspension that was stable for >1 day, we conclude that the hydration force provides the required repulsive energy barrier between particles to prevent the aggregation, which is a conclusion consistent with previous concepts reported in the literature (van Oss, 1997). Moreover, introducing $U_T = U_R + U_A + U_S$ in eq 3, the minimum hydration energy necessary to have $W \approx 10^{11}$ at 20 °C was estimated to be $\Delta G_{pwp} \approx 10.5$ mJ/m^2. At high temperatures, for partially unfolded globulins, the experimental values of half-life times are useful to estimate the stability ratio W. With the experimental value of $\bar{k}_a$ reported in Table 3, we found that $t_{1/2} = 7.4$ s at 80 °C; hence, $W \approx 10^6$. This result clearly indicates that the heat-induced aggregation process of globulins occurs as an RLA mechanism. In addition, one can estimate the value of ΔG_{pwp} required to give $W \approx 10^6$ at 80 °C. This calculation was also made from eq 3, and the result was $\Delta G_{pwp} \approx 7.9$ mJ/m^2. Thus, a reduction of $\sim$25% in ΔG_{pwp} is enough to break down the stability of the suspension. This decrease in ΔG_{pwp} is associated with both an increase of hydrophobic interaction energy and a decrease of the hydrophilic interaction energy, roughly quantified by eq 14.

Figure 6 presents our calculations of the interaction potential energy curves corresponding to the situations mentioned above. In this figure, the solid line refers to the potential energy of the DLVO theory, which predicts a repulsive peak of the order of $4k_BT$ at 20 °C. Also, the dashed line refers to the total potential energy U_T that includes the hydration interaction energy. This curve presents a repulsive peak of the order of $27k_BT$ at 20 °C. The dotted line is calculated at 80 °C by considering the interaction energy U_S necessary so that the particles aggregate in a few seconds. It is observed that the stability of the suspension is attained only with the help of the hydration forces. Also, the heat-induced denaturation affects the colloidal stability in our suspension

through the non-DLVO effects (hydration and hydrophobic forces).

Although the above calculations of W are rather consistent with the theory of colloidal suspension, there are several factors in our aggregating soy protein suspension that weaken the hypothesis of pair interaction used. In fact, one has to consider that doublets are formed only when a collision between denatured proteins occurs. The other native globulins present in the suspension screen the interaction between unfolded globulins. In addition, low degrees of denaturation involve low fractions of exposed hydrophobic sites of a globulin, which results in a weak attractive interaction. Under these conditions, it is clear that the aggregation process of partially unfolded soy proteins proceeds as an RLA. However, an exact calculation of W cannot be achieved in a simple manner.

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