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Light-Scattering Study of the Structure of Aggregates and Gels Formed by Heat-Denatured Whey Protein Isolate and β-Lactoglobulin at Neutral pH

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The structure of aggregates and gels formed by heat-denatured whey protein isolate (WPI) has been studied at pH 7 and different ionic strengths using light scattering and turbidimetry. The results were compared with those obtained for pure β-lactoglobulin (β-Lg). WPI aggregates were found to have the same self-similar structure as pure β-Lg aggregates. WPI formed gels above a critical concentration that varied from close to 100 g/L in the absence of added salt to about 10 g/L at 0.2 M NaCl. At low ionic strength (<0.05 M NaCl) homogeneous transparent gels were formed, while at higher ionic strength the gels became turbid but had the same self-similar structure as reported earlier for pure β-Lg. The length scale characterizing the heterogeneity of the gels increased exponentially with increasing NaCl concentration for both WPI and pure β-Lg, but the increase was steeper for the former.

KEYWORDS: WPI; β-lactoglobulin; aggregation; gels

INTRODUCTION

Whey proteins are widely employed in food formulations not only for their high nutritional value but also for their excellent functional properties, for example, gelation, and emulsion or foam formation. The major whey proteins are β-lactoglobulin (β-Lg), α-lactalbumin (α-La), and bovine serum albumin (BSA) (*1*), and their behavior upon thermal processing has been the subject of extensive research (*2*).

Heating aqueous solutions of whey proteins causes their denaturation and induces aggregation leading to gelation above a critical concentration (*3*−*5*). Pure α-La aggregates and gels much more slowly when heated than β-Lg or BSA, but in mixtures with either β-Lg or BSA it aggregates at rates comparable to those of the other proteins (*6*−*9*). In the latter case, mixed aggregates are formed through disulfide and other bonds (*8*, *10*−*13*).

The properties and structure of the gels are affected by medium composition and heat treatment (*14*−*16*). Transparent gels with a so-called fine-stranded structure are formed when the electrostatic repulsion is strong, i.e., at pH far from the isoelectric point of the proteins and low ionic strength (*3*, *17*). Under conditions of weak electrostatic repulsions, i.e., at high ionic strength and close to the isoelectric point of the proteins, opaque gels with a coarse, particulate structure are formed (*17*−*20*).

The structure of aggregates and gels of pure β-Lg (*21*−*26*), BSA (*27*−*29*) and ovalbumin (*26*, *30*−*32*) solutions has been studied in considerable detail at neutral pH using scattering techniques. It was found that the aggregates had a self-similar structure characterized by a fractal dimension close to 2 independent of the ionic strength. Transparent gels formed at low ionic strength were found to consist of relatively well-ordered strands of weakly branched proteins. These gels are transparent because they are homogeneous on length scales larger than a few tens of nanometers. At higher salt concentrations gels are more heterogeneous and consequently turbid. The latter type of gels could be described as an ensemble of "blobs" with the same self-similar internal structure as the aggregates.

To our knowledge, no detailed study has yet been made of the structure of WPI aggregates and gels using scattering techniques. The objective of the present work is to characterize the structure of WPI aggregates and gels formed at neutral pH and different ionic strengths and compare it with that of aggregates and gels formed by pure β-Lg. The aggregate structure for WPI turned out to be very similar to that of pure β-Lg. However, we observed a remarkable difference in the ionic strength dependence of the heterogeneity of the gels using a combination of light scattering and turbidimetry.

MATERIALS AND METHODS

Materials. Whey protein isolate (Prolacta90, batch no. 273) and β-lactoglobulin extracted from the same WPI batch were a kind gift from Lactalis Industrie (Laval, France). As stated by the manufacturer, the WPI powder had a composition of 97.8 wt % protein (71% β-Lg,

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18% α-La, 7% immunoglobulins, and 2% BSA), 2.0 wt % ash (dry weight basis), and 4.3 wt % moisture. The β-Lg powder contained less than a few percent of other proteins. All other chemicals used in this study were of analytical grade.

Preparation of Whey Protein Suspensions. Appropriate quantities of WPI and β-Lg powders were dissolved in Milli-Q water with 200 ppm NaN_3 added to avoid bacterial growth. The solutions were extensively dialyzed against Milli-Q water with 200 ppm NaN_3 and adjusted to pH 7. The ionic strength was set to the required value by adding aliquots of a concentrated NaCl solution. In the following we only consider the NaCl concentration, and it should be realized that the minimum ionic strength is 3 mM set by addition of NaN_3. For light-scattering experiments the samples were filtered through 0.2 μm pore size Anotope filters. The concentration was determined after filtration by UV adsorption at 278 nm using an extinction coefficient of 1.046 Lg^{-1} cm for WPI and 0.96 Lg^{-1} cm for β-Lg. Solutions in airtight light-scattering cells were heated at 80 °C for 24 h in a water bath. Heated samples were cooled on ice and stored at 20 °C until further analysis.

Determination of Residual Unaggregated Protein. The residual fraction of unaggregated protein was determined using gel filtration chromatography (GFC). The WPI and pure β-Lg samples were analyzed on a different apparatus. For WPI we used a TSK 2000 column SWXL (30 cm) (Tosohaas, Montgomeryville, PA) eluted with a solution containing 0.05 mM Tris and 0.1 M NaCl, and for pure β-Lg we used a TSK 2000 SW + 4000 SW column set (30 cm + 30 cm) in series with 0.1 M $NaNO_3$ as the elution liquid. The eluted protein was detected using UV absorption at 280 nm, and the amount of residual native protein was calculated as percent area relative to the area of unheated protein.

Light Scattering. Static (SLS) and dynamic (DLS) light-scattering measurements were made using an ALV-5000 multi-bit multi-tau correlator and a Spectra Physics solid-state laser operating with vertically polarized light with wavelength $\lambda = 532$ nm. The scattering wave vectors, q, varied from 3.0×10^{-3} to 3.5×10^{-2} nm^{-1} ($q = 4\pi n_s \sin(\theta/2)/\lambda$, with θ being the angle of observation and n_s the refractive index of the solution). The temperature was controlled by a thermostated bath to within ±0.1 °C.

The relative excess scattering intensity, I_r, was determined as the intensity minus the solvent-scattering intensity divided by the scattering intensity of toluene at 20 °C. The contribution of residual unaggregated protein to the intensity was negligible. In dilute solutions I_r is related to the weight-average molar mass (M_w) and the structure factor ($S(q)$) of the aggregate (*33*, *34*)

$$I_r = KCM_wS(q) \tag{1}$$

with C being the protein aggregate concentration and K an optical constant

$$K = \frac{4\pi^2 {n_s}^2}{\lambda^4 N_a}\left(\frac{\partial n}{\partial C}\right)^2\left(\frac{n_{tol}}{n_s}\right)^2\frac{1}{R_{tol}} \tag{2}$$

where N_a is Avogadro's number, $(\partial n/\partial C)$ is the refractive index increment, and R_{tol} is the Rayleigh constant of toluene at 20 °C ($R_{tol} = 2.79 \times 10^{-5}$ cm^{-1} at $\lambda = 532$ nm). n_{tol} and n_s are the refractive indices of toluene and the solvent, respectively. $(n_{tol}/n_s)^2$ corrects for the difference in the scattering volume of the solution and the solvent.

The z-average radius of gyration, R_{gz}, can be determined from the initial q dependence of $S(q)$

$$S(q) = \left[1 + \frac{q^2 {R_{gz}}^2}{3}\right]^{-1} \qquad qR_{gz} < 1 \tag{3}$$

Generally the structure of protein aggregates is self-similar over length scales between the radius of gyration and the size of the elementary building block (r_0). In that case $S(q)$ has a power law dependence on q

$$S(q) \propto q^{-df} \qquad {R_{gz}}^{-1} \ll q \ll {r_0}^{-1} \tag{4}$$

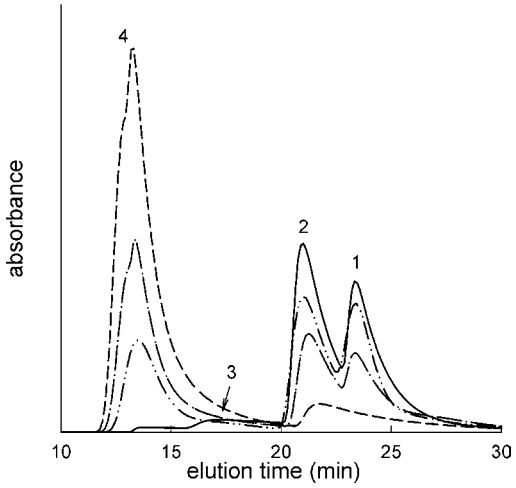

Figure 1. Gel filtration chromatography profiles of WPI solutions (5 g/L, pH 7, 0.1 M NaCl) unheated (solid line) and heated at 80 °C for 10 (dash-dot), 20 (long dash), and 65 (short dash) min. Peaks 1, 2, 3, and 4 correspond, respectively, to α-La, β-Lg, BSA and immunoglobulins, and protein aggregates.

where d_f is the so-called fractal dimension. d_f also characterizes the molar mass dependence of the radius of gyration for self-similar aggregates

$$M_w = aR_{gz}^{df} \qquad R_{gz} \gg r_0 \tag{5}$$

where the prefactor a depends on the molar mass of the elementary building block of the self-similar structure.

At higher concentrations, i.e., when interactions can no longer be neglected, $S(q)$ is the structure factor of the solution and M_w and R_{gz} should be replaced by an apparent molar mass (M_a) and radius (R_a). M_a is inversely proportional to the osmotic compressibility. If the interaction between the aggregates is repulsive, M_a decreases with increasing concentration, which is the case for the systems studied here. R_a is close to the correlation length of the concentration fluctuations, which characterizes the length scale beyond which the system is homogeneous. If the interaction is repulsive, the system becomes more ordered with increasing concentration and R_a decreases.

The turbidity (τ) was measured as a function of the wavelength (350−1100 nm) using a spectrometer and calculated using the following relation: $I_s/I_0 = \exp(-\tau l)$, with I_s being the light transmitted through the sample and I_0 the light transmitted through the solvent. The path length, l, was varied from 0.1 to 1 cm. The turbidity is related to the structure factor

$$\tau = K'CM_a\int_0^{2\pi}\int_0^{\pi} S(q)(1 + \cos^2\theta)\sin\theta\, d\theta\, d\varphi \tag{6}$$

where $K' = KR_{tol}(n_s/n_{tol})^2$.

RESULTS

Characterization of Native WPI. The elution profile of unheated WPI solution showed two main peaks that were attributed to α-La and β-Lg representing, respectively, 20% and 70% of the total amount of protein (see solid line in **Figure 1**). A small peak with 6% of the total area at smaller elution times represents BSA and immunoglobulins. The remaining 4% eluted in the form of aggregates. The presence of these proteins was confirmed by SDS-PAGE (data not shown).

Unheated WPI was further characterized using SLS and DLS. Although the fraction of aggregated protein in unheated WPI is very small, it strongly influences the light-scattering intensity. We corrected for this influence using dynamic light scattering as shown in ref 35 (*35*). At pH 7 the weight molar mass of

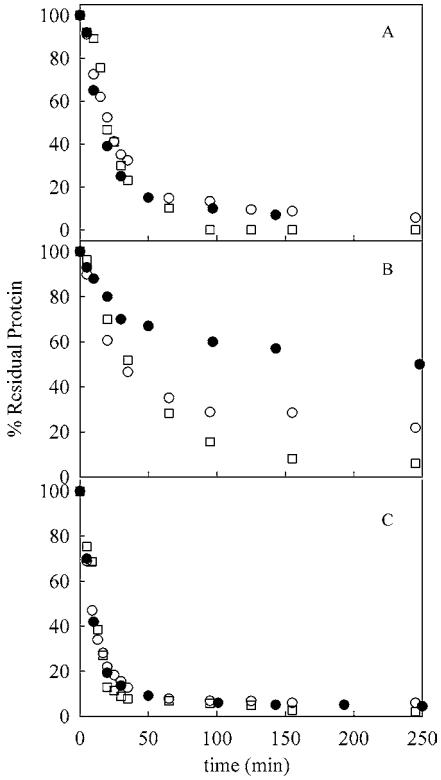

Figure 2. Residual fraction (% w/w) of unaggregated α-La (□) or β-Lg (○) as a function of heating time at 80 °C for WPI (open symbols) and pure β-Lg (filled symbols): (A) 5 g/L at 0.1 M NaCl; (B) 5 g/L without added salt, (C) 47 g/L without added salt.

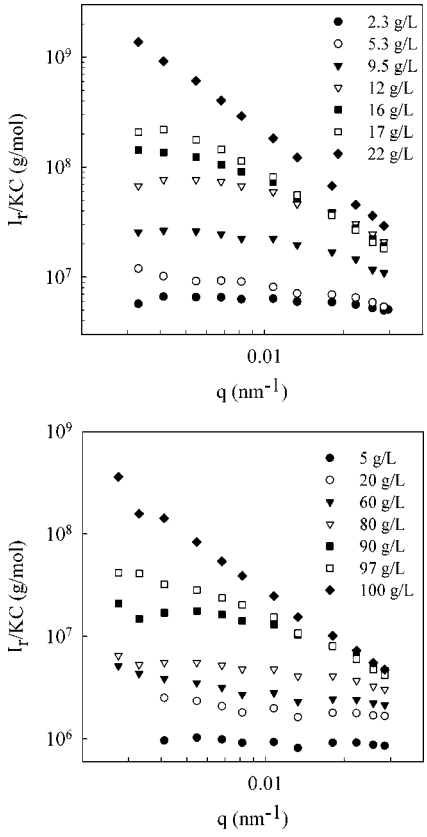

Figure 3. *q* dependence of I_r/KC for WPI aggregates formed after heating for 24 h at 80 °C at different protein concentrations indicated in the figures either with 0.1 M NaCl (top) or in the absence of added salt (bottom).

WPI was 44 kg/mol in the presence of 0.1 M NaCl, which is slightly higher than that of β-Lg dimers (36 kg/mol). Without added salt, M_w of WPI was lower, 26 kg/mol, due to the shift of the dimer−monomer equilibrium toward the monomer for β-Lg (*36*). The values of R_h showed the same tendency: 3 nm at 0.1 M NaCl and 2.3 nm without added salt.

Characterization of Heated WPI Using GFC. Figure 1 shows the effect of heating at 80 °C on the protein composition of 5 g/L WPI in the presence 0.1 M NaCl. The areas of the peaks representing native protein decreased progressively with increasing heating time, while a peak with increasing area representing aggregates appeared at small elution times. WPI formed rapidly large aggregates and very few oligomers, as found earlier for pure β-Lg (*21*) and WPI (*9*), and mixtures of α-La and β-Lg when β-Lg was the main component (*37*).

Figure 2 shows the decrease of the fraction of unaggregated α-La and β-Lg in WPI solutions as a function of the heating time at two ionic strengths and two protein concentrations. At the same concentration (5 g/L) aggregation of both β-Lg and α-La was considerably faster in the presence of 0.1 M NaCl than in the absence of added salt (compare **Figure 2A** and **2B**). Verheul (*38*) observed an increase of the reaction rate for β-Lg with increasing NaCl concentration up till about 0.1 M and a decrease at higher salt concentrations. The initial increase of the rate is probably due to screening of electrostatic repulsion. The subsequent decrease might be caused by an increased heat stability of the proteins. Increasing the WPI concentration by a factor of 10 in the absence of added salt led to a strong increase of the aggregation rate (compare **Figure 2B** and **2C**).

In all cases the aggregation rate was similar for β-Lg and α-La as reported earlier for mixtures heated at 80 °C (*6*−*9*). At $C = 5$ g/L, a significant amount (about 20%) of β-Lg did not aggregate in the absence of salt while most α-La did. This is a remarkable observation since, as mentioned in the Introduction, heat-denatured pure β-Lg aggregates much faster than pure α-La at the same conditions. For comparison we also show in **Figure 2** the decrease of the fraction of unaggregated protein in pure β-Lg solutions heated at the same conditions and the same total protein concentration. The aggregation rate was similar for WPI and pure β-Lg solutions. However, a notable difference was observed at $C = 5$ g/L in the absence of added salt. For pure β-Lg about 50% of the protein did not aggregate, while for WPI the fraction of unaggregated β-Lg remained at a lower value of about 20%. It was reported in ref *39* that β-Lg does not aggregate below a critical concentration that decreases with increasing ionic strength from 3 g/L in the absence of added salt to 0.03 g/L at 0.4 M NaCl. Apparently, in WPI the presence of α-La decreases the minimum association concentration for β-Lg.

Aggregate Structure. Soluble aggregates with different sizes were formed by heating WPI solutions at different protein concentrations for 24 h at 80 °C both in the absence of added salted and in the presence of 0.1 M NaCl. Large aggregates were only obtained close to the critical gel concentration; see below. Therefore, higher protein concentrations have been studied in the absence of salt than at 0.1 M NaCl. We verified that the growth of aggregates stagnated after this heating time. The amount of residual unaggregated protein was found to be negligible for the concentrations investigated. The aggregates were diluted so that interaction could be neglected.

Figure 3 shows the dependence of I_r/KC on the scattering wave vector for aggregates formed at 0.1 M NaCl and in the absence of added salt. With increasing protein concentration, the scattering intensity increased and its *q* dependence became more important because M_w and R_{gz} of the aggregates increased.

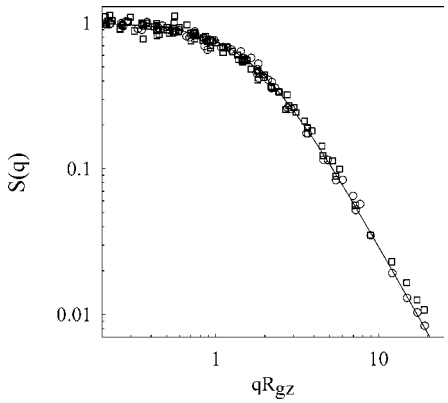

Figure 4. Comparison of the structure factors of highly diluted WPI aggregates formed after heating for 24 h at 80 °C at different concentrations either with 0.1 M NaCl (○) or in the absence of added salt (□). The solid line represents $S(q) = (1 + q^2R_{gz}^2/3)^{-1}$.

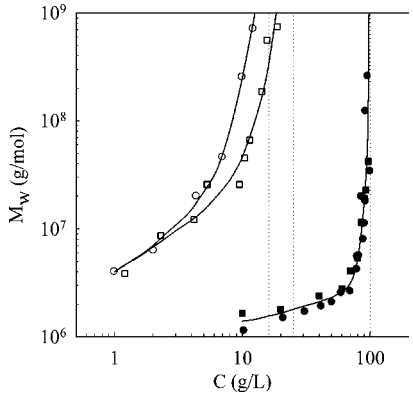

Figure 5. Concentration dependence of the weight-average molar mass of WPI (squares) and β-Lg (circles) aggregates formed in the presence of 0.1 M NaCl (open symbols) or in the absence of added salt (solid symbols). The solid lines are guides to the eye. The dotted lines indicate the critical gelation concentration: $C_g \approx 100$ g/L for WPI and β-Lg in the absence of added salt, $C_g \approx 25$ g/L for WPI, and $C_g \approx 15$ g/L for β-Lg at 0.1 M NaCl.

For the largest aggregates the intensity has a power law q dependence characteristic for self-similar clusters and the slope represents the so-called fractal dimension (d_f) (see Materials and Methods section). Similar results were reported earlier for pure β-Lg aggregates (*24*) formed at different concentrations of NaCl for which it was shown that the structure factor could be described by eq 4 over the whole q range covered by light scattering, implying that $d_f = 2$. However, in the absence of added salt, the structure factor deviated from eq 4 for large values of qR_{gz} and a slightly weaker power law decay was observed, indicating a slightly smaller fractal dimension: $d_f = 1.7$. The smaller fractal dimension implies that aggregates formed at low ionic strength had a more open structure.

Figure 4 shows that the results obtained for WPI at different concentrations superimposed when plotting $S(q)$ as a function of qR_{gz} and were also well described by eq 4. As was the case for pure β-Lg aggregates, a small deviation from eq 4 occurred at large qR_{gz} in the absence of added salt because the fractal dimension was slightly smaller. Within the experimental error the fractal dimensions of aggregates formed by WPI are the same as those formed by pure β-Lg both at 0.1 M NaCl ($d_f = 2.0 \pm 0.1$) and in the absence of added salt ($d_f = 1.7 \pm 0.1$)

Figure 5 compares the concentration dependence of M_w for WPI at both ionic strengths with that obtained for pure β-Lg solutions. In the absence of added salt, M_w was almost constant

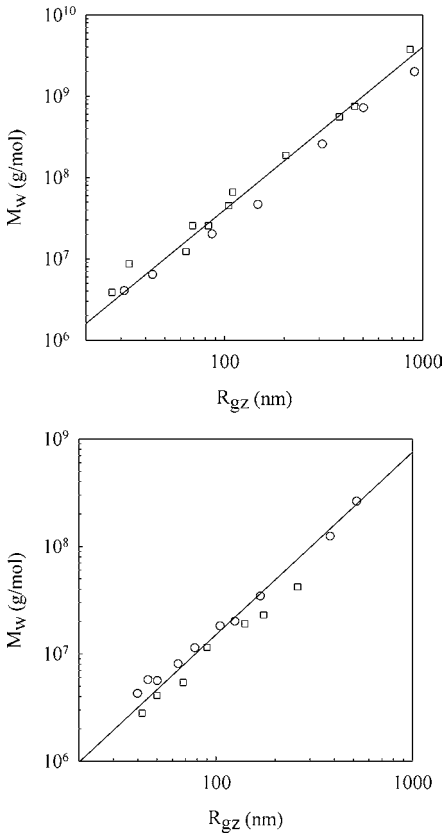

Figure 6. Dependence of M_w on R_{gz} for β-Lg aggregates (○) and WPI aggregates (□) formed after 24 h heating at 80 °C with 0.1 M NaCl (top) or without NaCl (bottom). The solid lines have slopes of 2.0 (top) and 1.7 (bottom).

at low protein concentrations but rose steeply when C approached the critical gelation concentration: $C_g \approx 100$ g/L for both systems, see below. At the gel point a system spanning aggregate is formed so that $M_w \rightarrow \infty$ as $C \rightarrow C_g$. In the presence of 0.1 M NaCl, M_w increased at much lower protein concentrations since $C_g \approx 25$ g/L for WPI and $C_g \approx 15$ g/L for pure β-Lg, The dependence of C_g on the ionic strength will be discussed below.

Figure 6 shows a comparison of the dependence of M_w on R_{gz} in the absence and presence of added salt for WPI and β-Lg aggregates. In all cases the dependence was compatible with a power law ($M_w \propto R_g^{d_f}$), which is expected for self-similar clusters. d_f obtained from **Figure 6** was consistent with that obtained from the structure factor and the same for WPI and pure β-Lg, but the experimental uncertainty was larger. The values of M_w for a given R_{gz} were slightly smaller for WPI than for β-Lg in the absence of added salt, while they were slightly larger in the presence of 0.1 M NaCl. The implication is that the local structure of the WPI aggregates is slightly denser than that of β-Lg aggregates in the presence of 0.1 M NaCl, while in the absence of added salt the local structure of β-Lg aggregates is slightly denser than that of WPI aggregates.

Gel Structure. As mentioned above, gels are formed only above a critical protein concentration. C_g was determined by visual observation after heating at 80 °C for 24 h by the absence of flow when tilting the samples and the presence of insoluble material after dilution. In this way C_g could be determined with a precision better than 10%. **Figure 7** shows the dependence of C_g on the NaCl concentration for WPI and β-Lg. At low ionic strength (<0.02 M), C_g was the same for β-Lg and WPI and weakly ionic strength dependent. At higher salt concentra-

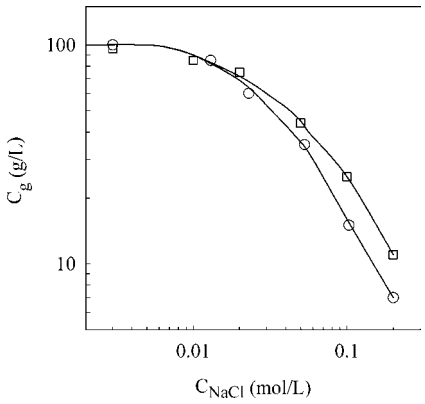

Figure 7. Dependence of C_g on the NaCl concentration for WPI (□) and β-Lg (○). The solid lines are guides to the eye.

tions, C_g decreased strongly for both systems but was somewhat larger for WPI.

We studied the influence of the ionic strength on the structure of WPI and β-Lg gels at a fixed concentration of 100 g/L. At low ionic strength the gels are transparent so that standard light-scattering techniques could be used. With increasing ionic strength the gels became more turbid and the measurements were influenced by multiple scattering. Nevertheless, the structure factor can still be determined in this case using the technique of cross-correlation dynamic light scattering (*40*). Elsewhere it was shown for pure β-Lg that the structure factor of turbid gels is the same as that of the dilute aggregates and can be described by eq 3 (*23*, *24*). Of course, one does not obtain M_w and R_{gz} but an apparent molar mass (M_a) and radius of gyration (R_a). As mentioned in the Materials and Methods section, M_a is proportional to the osmotic compressibility and R_a is close to the correlation length of the concentration fluctuations. Both parameters are a measure of the heterogeneity of the gels. The gels can be viewed as a close-packed ensemble of so-called blobs with radius R_a and molar mass M_a with the same self-similar structure as the aggregates. During heating the aggregates grow until they fill up the whole space. Further growth leads to interpenetration of the aggregates until a gel is formed. R_a is determined by the size of the aggregates when they fill up the space; see below. On length scales larger than R_a the gels are homogeneous.

If the structure factor is known, M_a and R_a can be determined more easily for turbid systems by measuring the turbidity as a function of the wavelength. Using $S(q) = (1 + q^2R_a^2/3)^{-1}$ in eq 6 the integral can be solved analytically

$$\tau = K'CM_a 2\pi\left[\frac{-4}{x^2}(x+2) + \frac{8x + 4x^2 + 8}{x^3}\ln(1+x)\right] \quad (7)$$

with

$$x = \left(\frac{4\pi n}{\lambda} \cdot \frac{R_a}{\sqrt{3}}\right)^2$$

We found in all cases that the wavelength dependence of τ was consistent with eq 7 if we included the small wavelength dependence of the refractive index increment: $(dn/dC) \propto \lambda^{-0.1}$. In the few cases where M_a could be determined both by light scattering and turbidimetry using different optical path lengths, the results were the same. Of course, if R_a is smaller than about 40 nm, $S(q)$ is close to unity over the relevant q range and τ is insensitive to the form of $S(q)$. In this case only M_a can be obtained from turbidity measurements. The maximum of M_a that can be determined using turbidity is limited by the transmission at the highest wavelengths with the minimum path length that could be used in the experiment (1 mm). The error on the values of M_a and R_a obtained from this method is less than 10% except for large M_a when the turbidity saturates at lower wavelengths and small R_a when there is little influence of the $S(q)$ on the wavelength dependence.

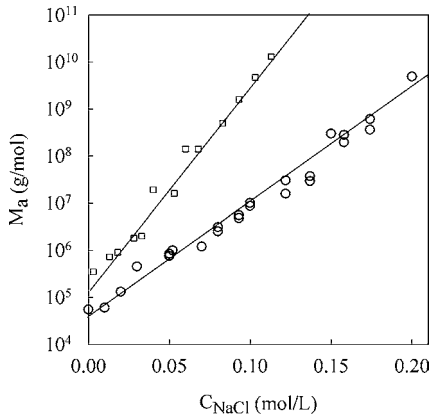

Figure 8. Dependence of M_a on the NaCl concentration for WPI (□) and β-Lg (○) solutions with $C = 100$ g/L after 24 h heating at 80 °C. The solid lines represent an exponential increase.

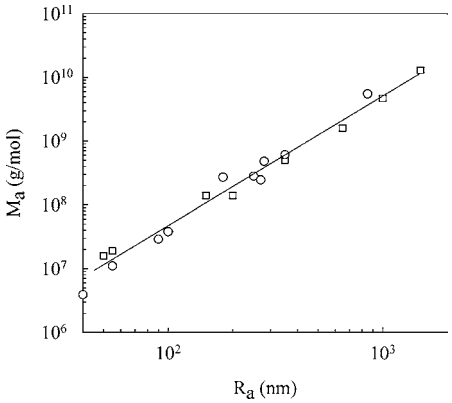

Figure 9. Dependence of M_a on R_a for WPI (□) and β-Lg (○) solutions with $C = 100$ g/L after 24 h heating at 80 °C. The solid line has a slope of 2.

Figure 8 compares the ionic strength dependence of M_a for WPI and β-Lg gels. In the absence of added salt, both systems are transparent but M_a is significantly larger for WPI gels than for β-Lg gels. An exponential increase of M_a with increasing salt concentration was observed for both systems, but for WPI gels the increase was steeper. It was shown elsewhere for β-Lg that the local density increased only weakly with increasing salt concentration (*23*). Therefore, the strong increase of M_a could only be caused by a strong increase of the heterogeneity of the gels. The steeper increase of M_a for WPI implies that at a given ionic strength WPI gels are coarser than β-Lg gels. The scatter of the data obtained from different samples is much larger than the experimental error for the same sample. The reason is probably that the structure is sensitive to small changes of the ionic strength and pH.

The dependence of M_a on R_a is shown in **Figure 9**. If one neglects the effect of the ionic strength on the local density, a power law dependence of M_a on R_a is expected: $M_a \propto R_a^{d_f}$. The results obtained at different salt concentrations can be approximately described by a power law with an exponent of 2, which demonstrates that the main reason for the increase of M_a is the increase of the heterogeneity of the gels.

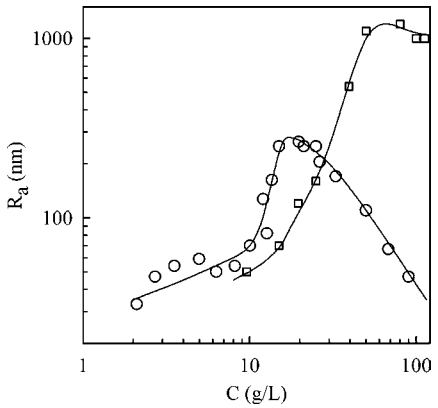

Figure 10. Concentration dependence of R_a for WPI (□) and β-Lg (○) solutions in the presence of 0.1 M NaCl after 24 h heating at 80 °C. The solid lines are guides to the eye.

So far we have discussed the dependence of the structure on the NaCl concentration at a given protein concentration. However, it was shown in ref *23* that the structure of heated protein solutions is strongly dependent on the protein concentration. Therefore, we performed a more detailed study of the concentration dependence at 0.1 M NaCl. The concentration dependence of R_a is shown in **Figure 10** for WPI and pure β-Lg. The results for pure β-Lg were discussed elsewhere (*24*) and show a maximum of R_a close to C_g. For both systems R_a increased initially with increasing protein concentration because larger aggregates were formed, see **Figure 5**, but the onset of the increase was shifted to higher protein concentrations for WPI. R_a reached a maximum of about 300 nm close to $C_g \approx$ 15 g/L for pure β-Lg, while for WPI it continued to increase above $C_g \approx 20$ g/L to a value of about 1 μm at 50 g/L.

In ref *23* it was shown for pure β-Lg that the dependence of M_a on R_a was the same as that of M_w on R_{gz}. We found that this was also the case for WPI (results not shown).

DISCUSSION

It has been established in quite some detail for β-Lg solutions at neutral pH that the aggregation process occurs in two steps (*41*). In the first step so-called preaggregates are formed containing approximately 100 monomers. The preaggregates have an elongated shape with a diameter of about 10 nm and a length of about 50 nm (*42*). In a second step these preaggregates associate to form large fractal clusters. At low ionic strength the second step is inhibited due to electrostatic interactions, which explains why M_w increases only weakly at low protein concentrations. From the present results for WPI we may conclude that a similar two-step mechanism occurs for WPI.

The structure of gels formed by heating pure β-Lg solutions at pH 7 was discussed in detail elsewhere (*23, 24, 26*). The main objective of the present study was to compare the structure of aggregates and gels formed by WPI with that of pure β-Lg. The protein aggregation rate was found to be the same for WPI and pure β-Lg because α-La co-aggregates easily with β-Lg even though it aggregates very slowly in pure solution. Indeed, almost all α-La aggregated even at $C = 5$ g/L in the absence of salt, when only one-half of the protein aggregated in pure β-Lg solutions. Recently, it was shown that the preaggregates are not formed at very low protein concentrations (*39*) and that the minimum concentration to induce aggregation decreases with increasing ionic strength. **Figure 2B** shows that the minimum association concentration for β-Lg is reduced in WPI due to the co-aggregation with α-La.

The overall structure of the aggregates is the same for WPI and β-Lg and characterized by a fractal dimension of 2.0 at 0.1 M NaCl and 1.7 in the absence of salt; the local structure is somewhat denser for WPI aggregates in 0.1 M NaCl and somewhat less dense in the absence of added salt. For both systems the aggregate size increases with increasing protein concentration and diverges at the critical gelation concentration. C_g is not sensitive to addition of very small amounts of NaCl, probably because the ionic strength is dominated by the contribution of the counterions. However, it decreases strongly above about 0.02 M NaCl. A similar NaCl concentration dependence of C_g was found for ovalbumin (*31*) and BSA (*29*). The decrease of C_g occurred at somewhat higher ionic strength for WPI than for β-Lg. The effect is equivalent to that of decreasing the NaCl concentration by a factor of 1.5. Apparently the preaggregates formed by pure β-Lg associate and gel more easily than those formed by WPI at higher ionic strengths.

The structure of the gels is very strongly dependent on the strength of the electrostatic interactions even though the aggregate structure itself is only weakly dependent on the NaCl concentration. It is important to distinguish the long-range repulsive interaction between the aggregates from the specific, short-range, attractive interaction between individual proteins that leads to bond formation. The latter causes aggregation and the increase of M_w, while the former reduces the concentration fluctuations and leads to a decrease of M_a compared to M_w.

It was shown using small-angle X-ray scattering that at low ionic strength the gels are ordered, showing a preferred distance between the branched protein strands (*26*). When more NaCl is added the electrostatic repulsion is screened, resulting in less order. As a consequence, the correlation length of the system increases with increasing NaCl concentration, but it is not obvious why the dependence is approximately exponential. When R_a is significantly larger than the radius of gyration of the preaggregates (about 25 nm) the structure factor can be described by eq 3. This was shown for β-Lg using cross-correlation dynamic light scattering (*23*). We did not determine $S(q)$ directly for WPI gels, but the wavelength dependence of the turbidity was consistent with values calculated from eq 6 using eq 3 for $S(q)$ both for β-Lg and WPI gels at all NaCl concentrations tested.

At low protein concentrations only small aggregates are formed during the heating process, but with increasing concentration more and larger aggregates are formed. They grow until they fill up the whole space and then connect to form the gel. In a first attempt to model the gelation process one may assume that the aggregates are monodisperse and interact through excluded volume interactions, i.e., the aggregates cannot interpenetrate. In this case, R_a and M_a are the size and the molar mass of the aggregates when they reach the overlap concentration. Simple geometric considerations give the following relationship between the protein concentration and M_a and R_a

$$C \approx \frac{3M_a}{N_a 4\pi R_a^3} \tag{8}$$

Using the power law relation between $M_a \propto R_a^2$ one obtains that $R_a \propto C^{-1}$. This was indeed found to be the case for pure β-Lg gels at 0.1 M NaCl (*24*).

However, it is obvious that this model cannot be valid in general because it implies that M_a and R_a are approximately independent of the ionic strength. Clearly, not only do the aggregates interact through excluded volume interaction, but other types of long-range interactions play a role that are

predominantly repulsive at low ionic strength and attractive at high ionic strength. Perhaps the agreement with the simple model for pure β-Lg gels at 0.1 M NaCl was caused by a fortuitous compensation of repulsive and attractive interactions. Increasing attractive interaction between the aggregates leads to increasing amplitude of the concentration fluctuations. One may still model the system as an ensemble of fractal blobs, but the ensemble is not monodisperse and the polydispersity increases strongly with increasing ionic strength. Apparently, the attraction is stronger between aggregates formed by WPI, leading to more heterogeneous gels at a given ionic strength.

If net attractive interaction dominates the excluded volume interaction, the system will phase separate. We did not observe phase separation in the range of NaCl concentrations studied, but microscopy has revealed the presence of dense protein domains in very heterogeneous gels formed close to the isoelectric point or in the presence of high salt concentrations (*20*, *43*). Unfortunately, the structure of such systems cannot be investigated using light scattering because they are too turbid.

Sometimes a distinction has been made between so-called fine-stranded (transparent) gels and particulate (turbid) gels based on electron microscopy. It is clear from the present investigation that there is a continuous transition from highly ordered, transparent gels to highly heterogeneous, turbid gels that is not compatible with this simple dichotomy. The transition between transparent gels and turbid gels that occurs at approximately 0.05 M NaCl for WPI and 0.1 M NaCl for pure β-Lg is simply the consequence of the strong, but continuous, increase of the scattering intensity. However, since the increase of the heterogeneity with increasing salt concentration is exponential, the transition between very homogeneous (finely stranded) and very heterogeneous (particulate gels) occurs over a small salt concentration range. Therefore, the dichotomy may be useful, but one should not conclude from this that there is a qualitative difference in the aggregation process at lower and higher ionic strength. The ionic strength at which the transition occurs depends on the pH and the type of protein, e.g., it occurs at somewhat lower NaCl concentrations for ovalbumin (*31*) and BSA (*29*) than for β-Lg at pH 7. It was shown here that it occurs at somewhat lower NaCl concentrations for WPI than for β-Lg. It would be interesting to investigate whether for other proteins and at different pH the increase of M_a and R_a with the NaCl concentration is also exponential.

The two main conclusions of the work presented here are as follows. (1) The aggregation mechanism of WPI at pH 7 is close to that of pure β-Lg. Heat-induced aggregation of WPI at neutral pH is a two-step process: formation of primary aggregates followed by their association into large self-similar aggregates with fractal dimension $d_f = 1.7$ in the absence of added salt and $d_f = 2$ at 0.1 M NaCl. (2) The heterogeneity of WPI and β-Lg gels as characterized by the correlation length increases exponentially with increasing salt concentration. The correlation length increased more strongly for WPI gels reaching 1 μm in the presence of 0.1 M NaCl than for β-Lg gels which reached 1 μm in the presence of 0.2 M NaCl.

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