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Molecular Mass Distributions of Heat-Induced β-Lactoglobulin Aggregates

Marion A. M. Hoffmann, Guido Sala, Cornelis Olieman, and Kees G. de Kruif*

Netherlands Institute for Dairy Research (NIZO), Kernhemseweg 2, 6718 ZB Ede, The Netherlands

The aggregates in heated bovine β-lactoglobulin solutions were separated by high-performance size-exclusion chromatography, and molecular characteristics were measured on-line using multiangle laser-light scattering detection (SEC-MALLS). This technique was proven to be a very useful and valuable approach for characterization of heat-induced β-lactoglobulin aggregates. Using TSK G2000 SW_{XL} and TSK G4000 SW_{XL} silica gel columns connected in series, aggregates up to a molecular mass of 4×10^6 Da could be separated, and complete molecular mass distributions were derived. Up to a molecular mass of 1.5×10^6 Da the molecular masses calculated by the MALLS agreed very well with the results obtained by conventional calibration. The measured molecular mass distributions of the heat-induced β-lactoglobulin aggregates varied strongly with experimental heating conditions (β-lactoglobulin concentration, pH, heating temperature, ionic strength). The results obtained with several β-lactoglobulin concentrations at neutral pH were consistent with a kinetic aggregation model based on thiol/disulfide exchange reactions. The average molecular mass and the radius of gyration of the heat-induced β-lactoglobulin aggregates increased with increasing initial β-lactoglobulin concentration.

Keywords: *β-Lactoglobulin; aggregation; size-exclusion chromatography; light scattering; molecular size*

INTRODUCTION

Whey proteins are important structural components in many foods and they are also used as ingredients because of their nutritional value and physicochemical properties (Kinsella and Whitehead, 1989; De Wit, 1990). These proteins are heat sensitive, and because almost all dairy processes involve the use of heat, a better understanding of the effect of heat on whey proteins in dairy foods is important. The thermal behavior of whey proteins is complex, involving conformational changes and subsequent aggregation. Under suitable conditions a gel also may be formed (Mulvihill and Donovan, 1987). Despite the extensive study of various aspects of whey protein denaturation and gelation, comparatively little work has been reported on the mechanism of aggregation and the quantitative characterization of aggregates and particles. In the food industry more detailed information on the effect of conditions on the size of the aggregates formed is desirable, to permit optimization of heat treatments that ensure the formation of specially sized and shaped aggregates, which give the desired organoleptic character to a product (Singer and Dunn, 1990). In this study we report on the size of bovine β-lactoglobulin (β-lg) aggregates, formed under various experimental conditions.

β-lg is the main protein in whey, constituting about 50% of the total whey protein in bovine milk. It is a globular protein, and the monomer (molecular mass = 18.3 kDa) consists of a single peptide chain of 162 amino acids and contains two intramolecular disulfide bonds and one free thiol group (McKenzie et al., 1972; Papiz et al., 1986). Under ambient conditions β-lg mainly exists as a noncovalently linked dimer, and by increasing the temperature the equilibrium is shifted toward the monomeric form (Georges et al., 1962). Upon heating above 50 °C the monomer at least partially unfolds and the inner hydrophobic parts of the molecule and the thiol group are exposed (McKenzie and Sawyer, 1967; Cairoli et al., 1994).

It is generally accepted that the thiol group and disulfide bonds play a role in the heat-induced aggregation and gelation of β-lg (Sawyer,1968; Watanabe and Klostermeyer, 1976; Hillier et al., 1980; Shimada and Cheftel, 1989; Liu et al., 1994; McSwiney et al., 1994; Iametti et al., 1995). In addition to chemical aggregation by covalent intermolecular disulfide bonds, aggregation by noncovalent interactions (ionic, van der Waals, hydrophobic) may also be involved. The extent of their relative contribution to the overall aggregation and gelation process is unclear (Mulvihill and Donovan, 1987; Shimada and Cheftel, 1989; McSwiney et al., 1994). Recently Roefs and De Kruif (1994) proposed a kinetic aggregation model based on thiol/disulfide exchange reactions leading to the formation of polydisperse, disulfide-linked aggregates. The model proposes, by analogy with polymer radical chemistry, an initiation, a propagation, and a termination reaction. In the initiation step (a first-order reaction) the thiol group is exposed and becomes reactive. The propagation reaction corresponds to the build-up of aggregates via thiol/disulfide exchange reactions between an activated β-lg intermediate, with a free, reactive thiol group, and a native, nonreactive β-lg molecule. In the termination step two active intermediates react and form a larger disulfide-linked polymer, without an exposed, reactive thiol group. The reaction scheme accounts for the formation of aggregates in which the monomers are linearly linked, but the aggregates are not stiff rods and may even have a spherical shape. The model holds for β-lg dissolved in water close to neutral pH and heated at relatively low temperatures (60 to 75 °C), and it gives a correct description of the decrease in concentration of native β-lg and the increase in scattered intensity, as measured by *in situ* light scattering, on the initial β-lg concentration (Roefs and De Kruif, 1994; Hoffmann et

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al., 1996). Furthermore, the kinetic model could be used to describe calorimetric measurements (Hoffmann et al., 1997).

The aim of this study was to characterize by high-performance size-exclusion chromatography (HP-SEC) the molecular masses of β-lg aggregates formed under well-defined conditions, where the kinetic predictions following from the model are valid. Collecting data in SEC involves measuring at each elution volume V, the molecular mass M, as well as a concentration-sensitive signal. Typically M is measured by calibrating columns with standards or by using an absolute instrument such as a light-scattering detector, and the concentration is determined with a refractive index (RI) or ultraviolet (UV) detector (Billingham, 1977; Shortt, 1993). However, with conventional calibration several problems may be involved, and in this investigation we probed the use of SEC with multiangle laser-light scattering detection (SEC-MALLS) for characterizing complete molecular mass distributions of heated β-lg solutions. The results obtained with this system were compared with conventional SEC, where the column was calibrated with proteins of known molecular mass. Furthermore, we also evaluated the effect of several experimental conditions (concentration, pH, temperature, and salt concentration) on the molecular mass distribution of the β-lg aggregates formed. The role of covalent disulfide bonds and noncovalent interactions in the formation of heat-induced aggregates was determined by treatment of heated solutions with 6 M urea (dissociates noncovalent aggregates linked via hydrogen bonds) or 6 M urea + 10 mM dithiothreitol (DTT) (dissociates disulfide linked aggregates) prior to analysis with the SEC-MALLS system.

MATERIALS AND METHODS

Materials. In all experiments we used a purified bovine β-lg sample, containing the genetic variants A and B (in a nearly 1:1 ratio), which was prepared at the pilot plant of NIZO from whey, basically following the procedure of Maubois et al. (1987). The sample contained 92% β-lg, 2% α-lactalbumin, 2% nonprotein nitrogen material, and 2.1% ash (including 0.73% Na^+, 0.02% K^+, 0.12% Ca^{2+}, 0.008% Mg^{2+}) on a dry mass basis. It contained 4% moisture (Hoffmann et al., 1996).

The protein was dissolved in double-distilled water (concentration range 10−100 g dry matter/L) and stirred for 2 h at room temperature. Unless otherwise stated the pH was near neutral (pH 6.8 ± 0.2). In separate experiments, the pH of a 10 g dry matter/L β-lg solution was set at pH 6.5 or pH 8.0 with 0.1 M HCl or 0.1 M NaOH. In the investigation of the influence of salt β-lg was dissolved in a 10 g of dry matter/L concentration in 0.1 M NaCl. After preparation all solutions were filtered (0.22 μm Millipore low-protein-binding filter, Bedford, MA). The solutions were stored at 4 °C and were used for further experiments within 24 h of preparation.

Heat Treatment. A series of test tubes each containing ca. 5 mL of a β-lg solution was heated in a water bath at 65 °C. After various time periods (2−48 h), a tube was taken and cooled in ice−water and then stored at 4 °C.

Determination of Concentration of Native β-lg. The concentration of native β-lg in the heated samples was determined by diluting the samples with double-distilled water to a concentration of about 2.5 g of native β-lg/L. The pH was adjusted to 4.7 ± 0.1 and the aggregates of denatured proteins were separated by centrifugation for 30 min at 20000g (De Wit, 1990). The native β-lg concentration present in the supernatant was determined by HP-SEC (Phenomenex column, Torrance, CA, type TSK G2000 SW_{XL}), with detection at 280 nm.

SEC-MALLS. The heated β-lg solutions were applied to a high-performance gel chromatography system, consisting of Phenomenex TSK G2000 SW_{XL} and TSK G4000 SW_{XL} silica gel columns (30 × 0.78 cm) in series. The exclusion limits of these two columns are 6.10^4 and 1.10^6 Da, respectively, for proteins. The columns were eluted with a phosphate buffer (6.956 g of KH_2PO_4, 6.956 g of K_2HPO_4, and 21.410 g of Na_2SO_4 in 1 L double distilled water, pH 6.5; buffer A) at a flow rate of 0.8 mL/min. Heated samples were diluted with double-distilled water to a β-lg concentration of 10 g of dry matter/L, and 0.01% (w/v) sodium azide was added to prevent microbial spoilage. These samples were further diluted with eluent to a final concentration of 1.0 g of dry matter/L. After filtration (0.22 μm Millipore low-protein-binding filter), 100 μL was injected into the chromatographic system. To determine the role of covalent and noncovalent interactions in the formation of aggregates, samples (concentration of 1.0 g dry matter/L) were also incubated with buffer B (buffer A containing 6 M urea; pH 6.8) or with buffer C (buffer B containing 10 mM DTT; pH adjusted to 8.3 with 1 M NaOH). Samples were incubated at room temperature for 1 h and 24 h in buffer B or C, respectively, and elution was done in buffer B.

For on-line light-scattering detection a DAWN-F MALLS photometer (Wyatt Technology, Santa Barbara, CA) was used, equipped with a K5 flow cell and a linearly polarized He−Ne laser-light source (5 mW) with wavelength $\lambda = 632.8$ nm. The DAWN contains 18 detectors, but the four smallest scattering angles (θ) were excluded, as the signal-to-noise ratio of these detectors was too low for an accurate measurement. Therefore, the range of wave vectors ($\mathbf{q}$) covered is $0.026 > \mathbf{q} > 0.0066$ nm^{-1}, with $\mathbf{q} = (4\pi n_s/\lambda)\sin(\theta/2)$, n_s being the solvent refractive index (Tanford, 1961). The concentration of eluting material was determined on-line with a UV spectrophotometer (LKB 2140 Rapid Spectral Detector, Bromma, Sweden) at 220 nm (buffer A) or 280 nm (buffer B) and a differential refractometer (ERC-7510 ERMA Optical Works Ltd, Tokyo, Japan). The data were accumulated and processed using Astra for Windows, version 4.0. Prior to the measurements the DAWN-F MALLS photometer was calibrated with filtered, HPLC-quality toluene and normalized using a bovine serum albumin solution. The molecular mass M_i and the mean square radius $\langle r^2 \rangle_i$ of material eluting in each slice i was calculated with a first-order Debye fit, using a specific refractive index increment (dn/dc) of 0.161 cm^3/g for buffer A and 0.131 cm^3/g for buffer B and a second virial coefficient (A_2) of zero.

The molecular masses of the β-lg aggregates were also determined by conventional calibration, using a Spectra-Physics SP4270 integrator (San José, CA) and standard proteins with a known molecular mass.

RESULTS AND DISCUSSION

Typical SEC-MALLS Elution Profiles. Figure 1 shows typical examples of elution profiles obtained with the TSK G2000/G4000 columns in combination with the MALLS photometer. For a 50 g/L β-lg solution, the elution profiles detected by UV and by MALLS at the 90° angle are shown for an unheated solution (Figure 1A) and a solution that has been heated for 24 h at 65 °C (Figure 1B). In the UV-trace of the unheated solution one peak is present with a retention volume of ≈20 mL. The MALLS software calculates a weight-averaged molecular mass (M_w) of (27 ± 2) × 10^3 Da for this peak, indicating that it is mainly dimeric β-lg. Upon heating, the native β-lg peak decreases with a peak assignable to the aggregates emerging and growing. After 24 h of heating (Figure 1B), some of the aggregates elute in the void volume (≈10 mL) of the columns and are not further separated, causing the small shoulder at the high molecular mass end of the aggregate peak in the UV- and the MALLS-trace. With most experimental conditions investigated, only a relatively small fraction of the aggregates was eluted in the void volume, and we deduced that these aggregates have molecular masses of 4 × 10^6 Da and higher (see below).

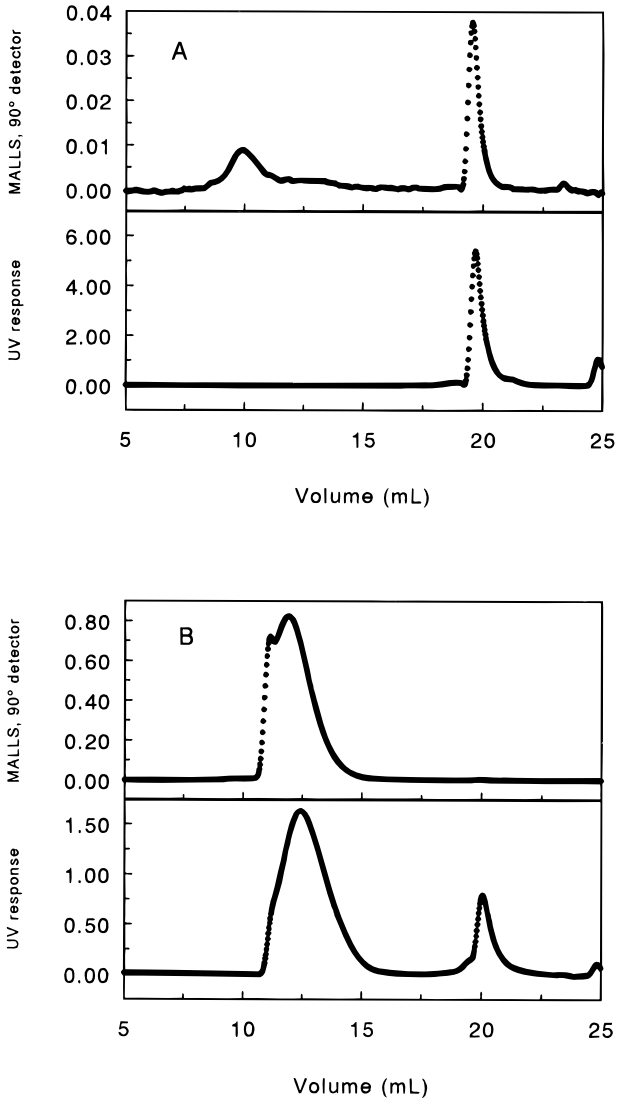

Figure 1. SEC elution profile of 50 g of dry matter/L of β-lg (A) unheated and (B) 24 h heated at 65 °C detected by UV (bottom) and MALLS at the 90° angle (top). Note the difference in *y*-axis scaling between A and B.

In the MALLS-trace of the unheated solution a peak with a retention volume of ≈10 mL can be seen. This peak was observed in all unheated solutions and is presumably caused by minute quantities of "impurities" such as dust. Since the light-scattering signal is proportional to the product of concentration and molecular mass, even a very small amount of a high-molecular-mass material is clearly visible. However, the aggregate peaks will not be significantly affected by these impurities (note the difference in the *y*-axis scaling of the MALLS signals in Figures 1A and B). The MALLS detector is much less sensitive to small-sized molecules, as can be seen from the β-lg peak in the 24 h heated sample. The residual native β-lg is observed in the UV-trace but not by the MALLS detector.

Comparison of the Molecular Masses Obtained by MALLS Detection and by Conventional Calibration. Technically, the molecular mass M_i of material eluting in slice i obtained from light-scattering measurements is weight-averaged, and the mean square radius $\langle r^2 \rangle_i$ is *z*-averaged (Tanford, 1961). However, assuming good chromatographic separation and using very small slices, we can assume that these values are to a good approximation the "true" molecular mass and mean square radius of the material eluting in that slice.

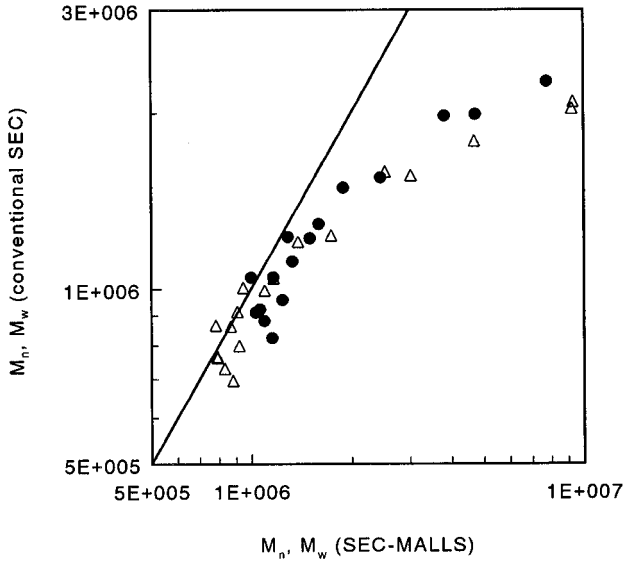

Figure 2. Comparison of number-averaged molecular masses (M_n) and weight-averaged molecular masses (M_w) determined by MALLS detection and by conventional calibration using TSK G2000 SW and TSK G4000 SW columns connected in series. The M_n (△) and M_w (●) values calculated with conventional calibration for the aggregate peak in a large number of analyzed β-lg solutions are plotted as a function of the corresponding values determined by MALLS detection.

The M_i values (obtained by MALLS detection or from a calibration curve) of each slice i can be used together with the concentrations c_i (measured with the UV- or RI-detector) to calculate the molecular mass moments (Billingham, 1977; Shortt, 1993). Figure 2 summarizes the number-averaged molecular masses (M_n) and the weight-averaged molecular masses (M_w) calculated for the aggregate peak in a number of heated β-lg solutions. The results obtained with classical calibration are plotted as a function of the corresponding values obtained with MALLS detection. For M_n and M_w values up to $\approx 1.5 \times 10^6$ Da conventional SEC and MALLS gave similar results, whereas for higher molecular masses conventional SEC gave an underestimation compared with the MALLS results.

When reporting the molecular size distribution of proteins as determined by SEC, it is necessary to keep in mind that elution is not necessarily governed by differences in molecular mass only. The gross shape of the molecule (including the hydration shell) and its physicochemical characteristics, especially net charge and hydrophobicity, may also play a role in separation (Billingham, 1977). Light scattering provides an absolute measurement of molecular masses whereas conventional calibration relies on a relation between molecular mass and retention time, which is valid only for molecules which are similar with respect to chemical structure and molecular conformation. Unfortunately, appropriate standards having the same composition and conformation as the β-lg aggregates are not available, and the β-lg aggregates may have a less compact and more expanded conformation than the globular standard proteins. Another problem with conventional calibration is that most β-lg aggregates were much larger than the largest standard protein (thyroglobulin, 669000 Da), and in order to encompass the whole molecular mass range of the β-lg aggregates we used a linear extrapolation of the calibration curve up to the exclusion limit of the columns. However, calibration curves usually show an upward curvature close to the exclusion limit (Billingham, 1977), and in this case the use of a linear

extrapolation results in an underestimation of the molecular mass of the material eluting close to the void volume. So, in general, conventional SEC is expected to give an underestimation of the molecular mass of material eluting in or close to the void volume, as can also be seen in Figure 2.

We also did some preliminary experiments with a Superose 6 column (Pharmacia LKB Biotechnology, Uppsala, Sweden, 30 × 1 cm, exclusion limit 4×10^7 Da for proteins). Although the exclusion limit of this column is much higher than that of the TSK G2000/G4000 columns, we noticed that with this chromatographic system a much larger fraction of the heat-induced β-lg aggregates was eluted in the void volume. This may indicate that with the Superose 6 column separation is probably not based on molecular size only but that specific interactions with the column (i.e. ionic exclusion effects) are involved. The molecular mass moments (M_n and M_w) determined by MALLS detection for the aggregate peak in the Superose 6 elution profiles were very close to the values obtained using the TSK G2000/G4000 columns, which supports the fact that the molecular masses calculated by the MALLS software are the absolute molecular masses, independent of the separating power of the columns used.

However, these absolute molecular masses may deviate from the "true" molecular masses. In the SEC-MALLS system two important parameters needed to obtain accurate molecular mass information are the refractive index increment (dn/dc) and the second virial coefficient (A_2). The dn/dc is necessary for determining the absolute quantities of material eluting at each volume increment. This information is needed for determining the weight fractions and for extrapolation of the light-scattering data to zero concentration in the Debye or Zimm plot. The dn/dc value was determined by assuming 100% mass recovery for unheated β-lg solutions of accurately known concentration. The value obtained in this way (0.161 cm^3/g) is relatively low compared with the value of 0.183 cm^3/g given by Perlmann and Longsworth (1948) at 632.8 nm. The lower value is probably due to the fact that SEC salts, which are coinjected with the β-lg sample, become separated from the protein and cause a RI peak at the total volume of the SEC columns used. Since the calculated molecular masses are proportional to 1/(dn/dc)^2, the use of the literature dn/dc value would give molecular masses which are 0.77 times those calculated with our value. This possible systematic deviation is relatively small compared with the differences (up to a factor 5) observed between molecular masses determined with classical calibration and with MALLS detection. A_2 describes the effective interaction between the protein particles. Given the low protein concentration of the material eluting in each volume increment we expect that by using $A_2 = 0$ only a small error has been made. Therefore, we think that the molecular masses determined by the MALLS are a good approximation of the "true" values.

Decrease in Concentration of Native β-lg. Upon heating, the concentration of native β-lg decreases. In our standard procedure the concentration of native β-lg in the heated solutions is determined by SEC analysis (TSK G2000 SW_{XL} column) of the acid soluble fraction (De Wit, 1990; Hoffmann et al, 1996). In this procedure the clear supernatant is analyzed, which is obtained after precipitation of the aggregates at pH 4.7 and contains only native β-lg (De Wit, 1990). In Figure 3 we compared the results of this SEC analysis of the acid soluble fraction with the fraction of native β-lg determined from SEC analysis of complete heated solutions (containing native and aggregated β-lg). The fraction of native β-lg in the complete heated solutions was calculated as mass eluted in the β-lg peak/total mass eluted (sum of β-lg and aggregate peak). With 10 g/L, the fraction of native β-lg determined from the relative peak areas in the TSK G2000/G4000 chromatograms is greatly overestimated compared with results obtained by SEC analysis of the acid soluble fraction, and the discrepancy increases with increasing heating time (Figure 3). This can be ascribed to the fact that with low β-lg concentrations a relatively large amount of small oligomers (disulfide linked dimers and trimers) are formed, which with SEC analysis of the complete heated solutions are seen as "native" β-lg. With SEC of the acid soluble fraction, all aggregates, including these small aggregates, have been precipitated during the pretreatment of the sample and, as such, are not determined as native β-lg. With higher initial β-lg concentrations, mainly larger aggregates are formed and only a very small fraction of these small oligomers, so with 30 g/L β-lg the values obtained with the two techniques become closer, and for 50 g/L (and higher concentrations, results not shown) no more differences are observed. Similar effects were observed with sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) analysis (8−25% gels) of heated β-lg solutions (Hoffmann and Van Mil, 1997), where with 10 g/L β-lg we observed the formation of small oligomers of β-lg (di-, tri-, and tetramers) in addition to high-molecular-mass aggregates ($M > 3 \times 10^5$ Da) on top of the gel. With 50 g/L only very faint small aggregate bands were observed, and mainly large aggregates on top of the stacking and separation gel could be seen.

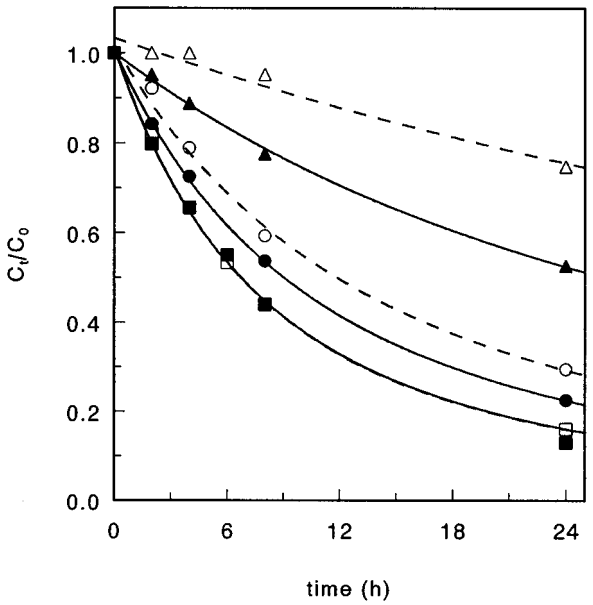

Figure 3. Comparison of the fraction of native β-lg (C_t/C_0) determined from the TSK G2000/G4000 chromatograms of the complete heated solutions (calculated as mass eluted in the β-lg peak/total mass eluted) and with SEC analysis after precipitation of the aggregates at pH 4.7. The values calculated from TSK G2000/G4000 chromatograms (open symbols) and with SEC analysis of the acid soluble fraction (closed symbols) are shown for three different initial β-lg concentrations: (▲,△) 10, (●,○) 30, and (■,□) 50 g of dry matter/L. The drawn lines represent curves with order 1.5 reaction kinetics fitted to the experimental points.

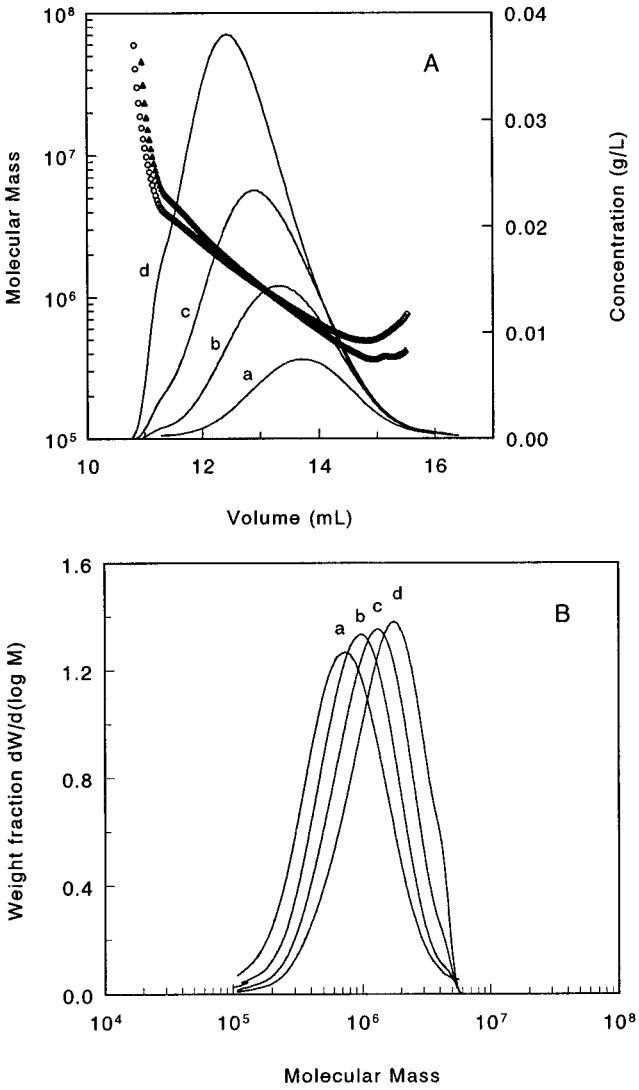

Figure 4. (A) SEC-UV elution profiles of the aggregate peak obtained for 50 g of dry matter/L of β-lg solutions heated for several time periods at 65 °C: (a) 2, (b) 4, (c) 8, and (d) 24 h. For the 4 h (▲) and 24 h (○) heated solutions the elution profiles are overlaid with the calculated molecular masses. (B) Weight fraction plots constructed from the aggregate peaks in Figure 4A.

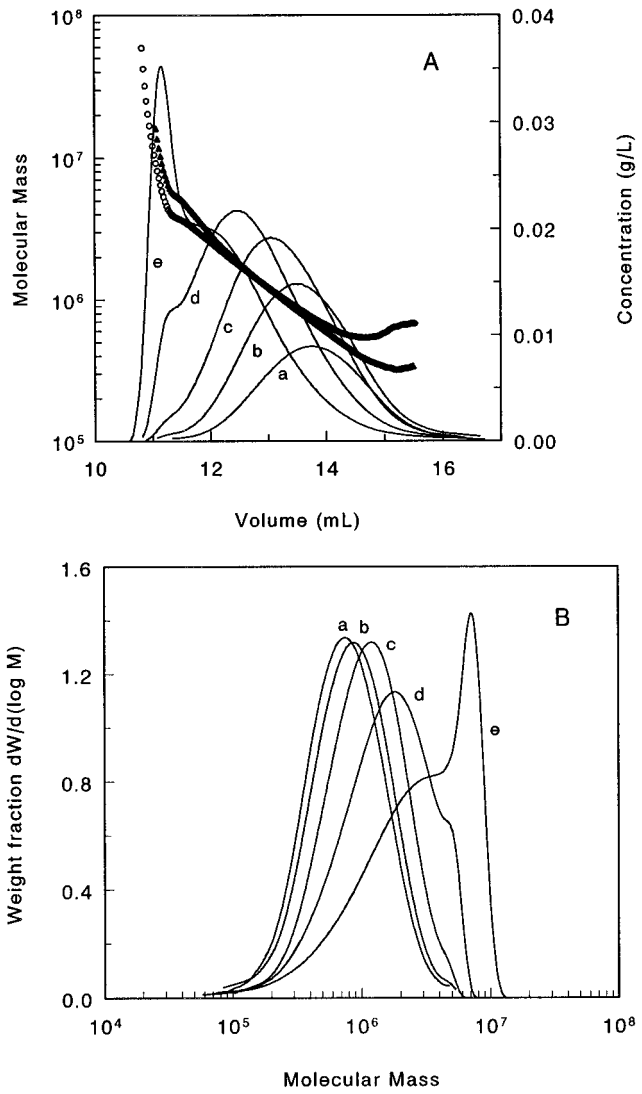

Figure 5. (A) SEC-UV elution profiles of the aggregate peak obtained for several β-lg concentrations, heated for different time periods at 65 °C such that ≈50% of native β-lg was aggregated ($C_t/C_0 \approx 0.5$, determined with SEC analysis of the acid soluble fraction): (a) 10, (b) 30, (c) 50, (d) 75, and (e) 100 g of dry matter/L of β-lg. For 30 g/L (▲) and 75 g/L (○) the elution profiles are overlaid with the calculated molecular masses. (B) Weight fraction plots constructed from the aggregate peaks in Figure 5A.

Effect of Heating Time And initial β-lg Concentration on Aggregate Formation. With increasing heating time more native β-lg is converted into aggregates (Figure 3). The molecular masses of the aggregates formed in 50 g/L β-lg solutions that had been heated for various times at 65 °C were determined with the TSK G2000/G4000-MALLS system. Figure 4A shows the SEC-UV elution profiles of the aggregate peak in these solutions, and for two solutions these profiles are overlaid with the calculated molecular masses. In general, in the central part of all aggregate peaks a linear relationship between log *M* and elution volume was observed, which corroborates the separation power of the chromatographic system used. At the extremes, deviations from this linear relationship occur. The deviation at the high-molecular-mass end of the distribution is caused by the fact that these particles elute in the void volume (molecular masses larger than $\approx 4 \times 10^6$ Da). The deviation at the low-molecular-mass end is presumably caused by a combination of a lower sensitivity of the MALLS detector in this range and the very small amounts of material present in the low-molecular-mass tail. The MALLS software calculates the molecular mass in each slice from the quotient of the Raleigh ratio and the concentration in that slice, and for low concentrations the determined molecular masses will be imprecise and highly sensitive for the allocation of the base line. In the calculation of molecular mass distributions the MALLS software uses a linear extrapolation of the linear part of the log *M* versus elution plot in the central part of the peak to lower and higher molecular masses. With 50 g/L β-lg these distributions show a small shift toward higher molecular masses with increasing heating time (Figure 4B).

For evaluating the effect of initial β-lg concentration on the size of the formed aggregates, we compared solutions (10−100 g/L β-lg) that had been heated for different times at 65 °C, such that for all concentrations about the same fraction of native β-lg was converted into aggregates. Figure 5A shows TSK G2000/G4000-MALLS elution profiles of solutions in which 50% of native β-lg is aggregated (determined with SEC analysis of the acid soluble fraction). The UV profiles show that

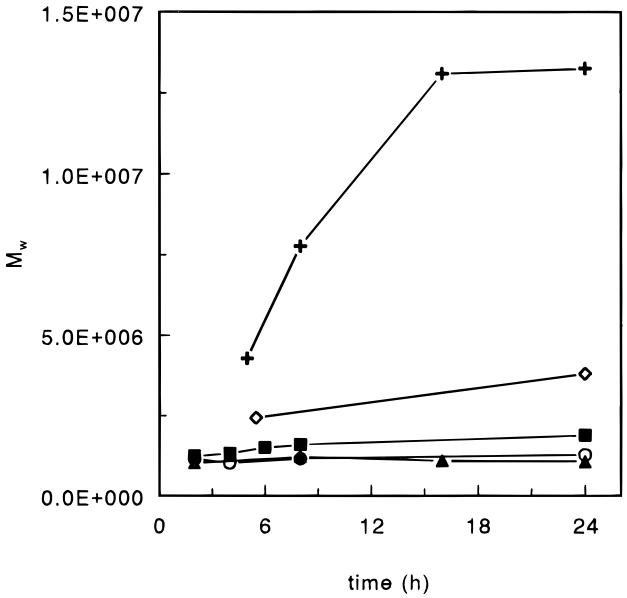

Figure 6. Weight-averaged molecular masses (M_w) calculated for the aggregate peak of heated β-lg solutions as a function of heating time: (▲) 10, (○) 30 (■) 50, (◊) 75, and (+) 100 g of dry matter/L of β-lg.

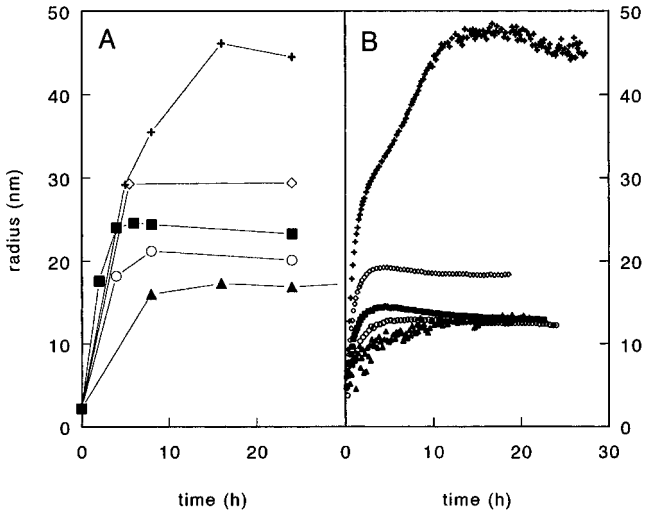

Figure 7. (A) *z*-Average radius of gyration determined with the TSK G2000/G4000-MALLS system for the aggregate peak in heated β-lg solutions and (B) the apparent Stokes−Einstein radius determined by *in situ* dynamic light-scattering experiments at 65 °C as a function of heating time for several initial β-lg concentrations: (▲) 10, (○) 30 (■) 50, (◊) 75, and (+) 100 g of dry matter/L of β-lg.

with increasing initial β-lg concentration the aggregate peak shifts toward lower elution volumes, i.e. higher molecular masses. With 100 g/L β-lg a substantial part of the aggregate peak elutes in the void volume. The weight fraction of the aggregates (Figure 5B) shifts toward higher molecular masses with increasing initial β-lg concentration, demonstrating that the average size of the aggregates increases with increasing concentration, as predicted by Roefs and De Kruif (1994). This shift toward higher molecular masses is much stronger than the shift which was observed for one β-lg concentration as a function of heating time (Figure 4B).

Figure 6 summarizes weight-averaged molecular masses (M_w) calculated for several β-lg concentrations as a function of heating time. For accurate determination of molecular masses the concentration signal has to be high enough. Once a reliable concentration signal for the aggregate peak was obtained, and hence, an M_w value could be calculated, this value remained about constant or showed a small increase upon prolonged heat-treatment. Only for the highest concentration (100 g dry matter/L) was a different behavior observed: M_w continued to increase with increasing heating time and only after ≈16 h of heating, when less than 5% of the initial native β-lg concentration was left, was a plateau level reached. For the number-averaged molecular mass (M_n), similar effects were observed. These effects are in line with the effect of heating time on the *z*-average radius of gyration of the aggregates formed (Figure 7A). For the unheated solutions, no accurate radius of gyration moments could be calculated, as they were below the detection limit of the MALLS (10 nm). The *z*-average radius of gyration of these solutions was "set" at 2.16 nm, which is the literature value given for the radius of gyration of the dimer (Witz et al., 1964). With 10−75 g/L β-lg after 4−8 h of heating a plateau level was reached, whereas with 100 g/L no plateau level was reached within that time, and the radius of gyration increased with heating time, reaching a constant level only after ≈16 h. However, here it has to be remarked that for an accurate determination of the radius of gyration this value should be larger than 1/20 times the wave length of the laser-light source used for light-scattering measurements (Tanford, 1961). As most calculated radii were below this limit (≈30 nm), the error in the values plotted in Figure 7A may be quite large.

In Figure 7B also the apparent Stokes−Einstein radius, measured by *in situ* dynamic light-scattering experiments at 65 °C (Hoffmann et al., 1996), is shown as a function of time. Although the apparent Stokes−Einstein radius and the radius of gyration cannot be compared directly they clearly show the same trend as a function of time. These results demonstrate that upon heating of β-lg concentrations in the range 10−75 g/L, particles of a constant average size are formed and, although during prolonged heating more native β-lg is converted into aggregates, the molecular mass distribution remains about the same, as predicted by the model of Roefs and De Kruif (Roefs and de Kruif, 1994; Hoffmann et al., 1996). The increase in particle size with heating time which is observed with 100 g/L β-lg can be ascribed to the Trommsdorff effect (Roefs and De Kruif, 1994). This effect is frequently encountered in polymer chemistry (Hiemenz, 1984; North, 1966; Mita and Horie, 1987) and is caused by an increased viscosity of the reaction medium as the reaction proceeds, due to the formation of very large aggregates. Especially the diffusional and rotational motion of long reactive intermediates will be affected, and this leads to an acceleration of the overall aggregation reaction and to an increase in aggregate size with time.

The polydispersity (M_w/M_n) of the heat-induced β-lg aggregates was found to increase slightly with increasing initial β-lg concentration and varied between 1.33 ± 0.03 (10 g/L β-lg) and 1.5 ± 0.1 (100 g/L β-lg). These values are close to the theoretical value ($M_w/M_n = 1.5$) calculated for free-radical polymerization reactions (Tanford, 1961). For all concentrations no significant effect of heating time on the polydispersity index was observed.

The reproducibility of the SEC-MALLS system used was tested by multiple injection of the same sample on various days. In general the values for M_n, M_w, polydispersity, and the radius of gyration only varied by 5−10% between the different runs. If we compared

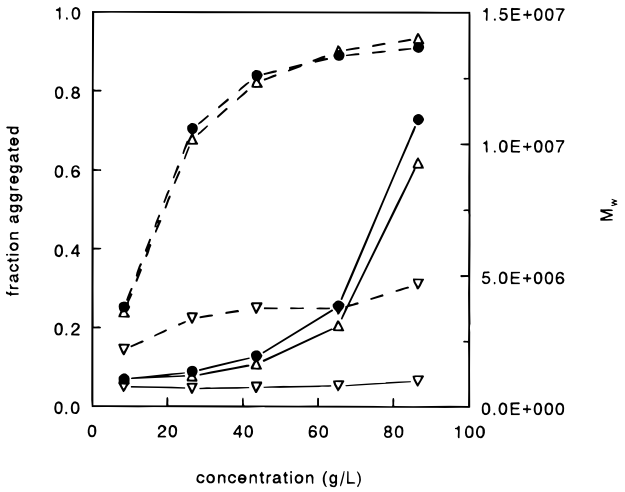

Figure 8. Effect of treatment in 6 M urea and in 6 M urea + 10 mM DTT (pH 8.3) prior to analysis of heated β-lg solutions (24 h, 65 °C). The fraction of aggregated β-lg (based on the peak area of the aggregate peak in the TSK G2000/G4000 chromatograms) (dashed lines) and the weight-averaged molecular masses (M_w) calculated for the aggregate peak (full lines) are shown for samples treated in three different ways: (●) no pretreatment, analysis in phosphate buffer, pH 6.5 (= buffer A); (△) incubated for 1 h in buffer A containing 6 M urea (= buffer B, pH 6.8), analysis in buffer B; (▽) incubated for 24 h in buffer B containing 10 mM DTT (pH 8.3), analysis in buffer B.

solutions of different heating experiments, somewhat larger differences were found (up to 20%), but as a function of concentration and heating rate the same trends, as reported here, were observed. In order to investigate whether further polymerization occurs after diluting the samples with eluent we analyzed solutions several times after the dilution with eluent. For all concentrations the chromatographic patterns did not show any differences compared with the sample directly applied, indicating that no further polymerization reactions occurred in the eluent or during the chromatographic procedure.

From a double logarithmic plot of radius of gyration versus molecular mass a shape factor can be determined. However, since for most experimental conditions the radii of gyration were below or close to 30 nm, in general the slopes of these plots were very small (≤0.25). Although no consistent shape factors could be obtained, the low values may indicate that the aggregates formed have a more or less spherical conformation.

Interactions Involved in Aggregate Formation. To elucidate the role of covalent intermolecular disulfide bonds and noncovalent interactions in the formation of aggregates, heated β-lg solutions were incubated with 6 M urea or 6 M urea + 10 mM DTT prior to elution in phosphate buffer containing 6 M urea. The effect of these treatments on the fraction of aggregated material and the weight-averaged molecular mass of the aggregates in β-lg solutions that had been heated for 24 h at 65 °C is reported in Figure 8. Treatment with 6 M urea had little effect on the fraction of aggregated material (based on the peak area of the aggregates relative to the total peak area). However, in the chromatograms two or three small shoulders at the high-molecular-mass end of the native β-lg peak appeared. The M_w values calculated for these peaks were in the range $2 \times 10^4 - 3 \times 10^5$ Da, indicating that they are small oligomers of β-lg. These shoulders could also be observed in the unheated samples, demonstrating that these oligomers were not formed due to the dissociation of high-molecular-mass aggregates in 6 M urea but were formed from native β-lg. McKenzie (1971) and McKenzie and Ralston (1973) reported that in the presence of urea complex, irreversible unfolding and aggregation reactions occurred. At pH 7.0 irreversible oligomeric products were formed by thiol/disulfide exchange reactions and to a lesser extent from thiol oxidation.

Upon treatment in 6 M urea a small decrease in the M_w value calculated for the aggregate peak was observed, and this effect increased with increasing initial β-lg concentration. Given the fact that the total amount of aggregated material did not change in the presence of 6 M urea, this could only be ascribed to the dispersion of a small fraction of high-molecular-mass aggregates into smaller aggregates. This indicates that some (primary) disulfide linked aggregates had formed larger aggregates via noncovalent interactions. These secondary aggregation reactions seem to become increasingly important with increasing β-lg concentration.

After incubation with 6 M urea + 10 mM DTT, a considerable decrease both in the amount of aggregated protein and in M_w of the aggregate peak occurred, demonstrating that a large proportion of high-molecular-mass aggregates were formed by intermolecular disulfide bonds. However, no complete dissociation of the aggregates into monomers/dimers was observed. This may be due to the fact that the resulting aggregates (M_w values in the range $7-10 \times 10^5$ Da) are very compact, with the monomers being tightly incorporated, and that as such the intermolecular disulfide bonds are not accessible to DTT. Another explanation might be that partial reduction of intramolecular disulfide bonds enhances interactions between exposed hydrophobic regions and generates extra thiol groups which may participate in thiol/disulfide exchange reactions (Shimada and Cheftel, 1989). The treatment with DTT appeared to be very sensitive to the DTT concentration used and the pH of the reaction medium, and under some experimental conditions we observed an increase in the aggregate peak, compared with the untreated sample. Also, in the unheated samples, aggregates were observed (results not shown). Under the experimental conditions used in this investigation (10 mM DTT, pH 8.3), no aggregates were observed in the unheated samples, but it cannot be excluded that some of the aggregates, observed in the heated samples after treatment, had been induced by DTT and that these were not breakdown products of high-molecular-mass disulfide-linked aggregates. The results obtained clearly illustrate the role of intermolecular disulfide bridges in the formation of heat-induced β-lg aggregates and show that in water near neutral pH noncovalent interactions are of minor importance.

Effect of pH, Heating Temperature, and Salt on Aggregate Formation. For 10 g/L β-lg solutions we investigated the effect of different experimental conditions on the weight distributions of the aggregates formed after 24 h of heating at 65 °C. Figure 9A shows that the aggregation of β-lg is very sensitive to small variations in pH. The aggregates formed at pH 6.5 have a much higher average molecular mass compared with those formed at neutral pH (pH 7.0), although less native β-lg has reacted. At pH 8.0 more native β-lg has been converted into aggregates. However, under these conditions many very small aggregates are formed, which elute in a shoulder at the high-molecular-mass

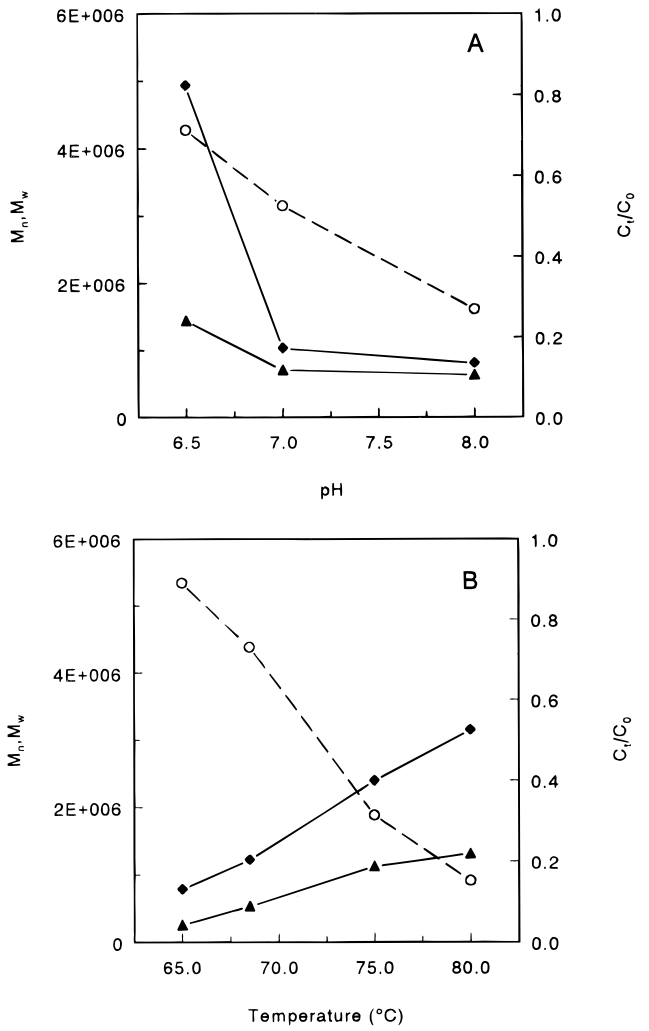

Figure 9. Number-averaged molecular masses (▲) and weight-averaged molecular masses (◆) calculated with the SEC-MALLS system for the aggregate peak of 10 g/L β-lg solutions heated under different experimental conditions: (A) M_n and M_w as a function of pH for solutions that have been heated for 24 h at 65 °C and (B) M_n and M_w as a function of heating temperature (heated for 2 h, pH 7.0). Also the fraction native β-lg (C_t/C_0, determined with SEC analysis of the acid soluble fraction) is indicated (○).

end of the native β-lg peak, and only a small fraction with a molecular mass $>1 \times 10^5$ is formed. The M_n and M_w values in Figure 9A were calculated for this high-molecular-mass fraction, and if we also include the oligomeric aggregate products, much lower values would be obtained for the solutions heated at pH 8.0. The effect of pH on the molecular mass distribution of the aggregates formed is in line with the results from previous gel-electrophoresis and light-scattering studies (Hoffmann et al., 1996; Hoffmann and Van Mil, 1997). A more extensive study of the effect of pH is currently being undertaken, the results of which will be published at a later stage.

The addition of salt had a pronounced effect on the molecular mass distribution of β-lg aggregates. After heating 10 g/L β-lg in 0.1 M NaCl for 24 h at 65 °C, very large aggregates were formed which were almost completely eluted in the void volume. These results are consistent with the findings of Eloffson (1996), who used a TSK G4000 SW column in combination with MALLS detection for the determination of average molecular masses of aggregates formed with β-lg A and β-lg B in 0.1 M NaCl at 68 °C. Since in the presence of salt very large aggregates are formed (Verheul et al., 1995) and even after relatively short heating times part of the aggregates are completely excluded, it is difficult to analyze quantitatively the aggregate distribution with the SEC columns used. Therefore, the effect of salt was not further investigated in this study.

The average molecular masses of the aggregates formed after heating for 2 h at different temperatures were found to increase with increasing heating temperature (Figure 9B). This may be due to the fact that more native β-lg has been converted into aggregates. However, if we compare solutions that have been heated for different times at the various temperatures such that the same fraction of native β-lg had reacted, M_w also was found to increase. For example, with solutions heated for different times at 65 and 75 °C such that $\approx$70% of native β-lg had reacted (i.e. $C_t/C_0 \approx 0.3$), M_w was found to increase from 1×10^6 to 2.5×10^6 Da. The *z*-average radius of gyration of the aggregates formed after 2 h of heating increased only slightly with increasing heating temperature: from 20.1 nm at 65 °C to 23.3 nm at 80 °C.

Conclusion. We have shown that size-exclusion chromatography in combination with laser-light scattering for determination of molecular masses offers a very useful and valuable approach for studying the size of β-lg aggregates formed under different experimental conditions. Compared with conventional SEC, where the column is calibrated with proteins with a known molecular mass, the SEC-MALLS system has several advantages. The light-scattering measurements automatically provide a column calibration for every sample, thereby obviating the need for time-consuming, conformation-dependent calibration procedures. In particular, in cases where the calibration curve has to be extrapolated because no suitable standards are available (as in this investigation), it may be very useful because MALLS calculates absolute molecular masses, which are not affected by the separating power of the column.

The results obtained in this investigation are qualitatively in good agreement with information from earlier light-scattering and gel-electrophoresis studies in our laboratory (Hoffmann et al., 1996; Hoffmann and Van Mil, 1997). However, with light-scattering only a weighted size average of all aggregates was seen, and this size average was strongly dominated by the size of the largest aggregates in the system. With gel electrophoresis only qualitative information on the size of the formed aggregates was obtained, and especially for the larger aggregates ($>10^5$ Da) the separation was much less compared with the separating power of the chromatographic system used in this study. Compared with these two techniques, SEC-MALLS has proven to be much more useful because in addition to studying qualitatively the effect of different experimental conditions on the molecular masses of the aggregates also quantitative information can be obtained and complete molecular mass distributions of the β-lg aggregates can be derived.

ABBREVIATIONS USED

HP-SEC, high-performance size-exclusion chromatography; MALLS, multiangle laser-light scattering; RI, refractive index; UV, ultraviolet; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; M_n, number-averaged molecular mass; M_w, weight-averaged molecular mass.

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