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Effects of Flow on Hen Egg White Lysozyme (HEWL) Fibril Formation: Length Distribution, Flexibility, and Kinetics

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The effect of steady shear and turbulent flow on the formation of amyloid fibrils from hen egg white lysozyme (HEWL) was studied. The conversion and size distribution of fibrils obtained by heating HEWL solutions at pH 2 were determined. The formation of fibrils was quantified using flow-induced birefringence. The size distribution was fitted using decay of birefringence measurements and transmission electron microscopy (TEM). The morphology of HEWL fibrils and the kinetics of their formation varied considerably depending on the flow applied. With increasing shear or stirring rate, more rod-like and shorter fibrils were obtained, and the conversion into fibrils was increased. The size distribution and final fibril concentration were significantly different from those obtained in the same heat treatment at rest. The width of the length distribution of fibrils was influenced by the homogeneity of the flow.

KEYWORDS: Lysozyme; fibrils; heat-induced aggregation; conversion

INTRODUCTION

The assembly of food-grade proteins, such as β-lactoglobulin, bovine serum albumin, and lysozyme (LSZ) from hen egg white (HEWL), into fibrils (*1*−*4*) has recently gained considerable attention, because of the potential for broadening the functional properties of these proteins (*5*). In a recent paper, fibrils were used to develop microcapsules with a fibril-reinforced nanocomposite shell (*6*). Fibrils are also suitable for use as efficient thickening or gelling agents (*7*−*9*). For use in encapsulation and other functional applications, a simple but controlled method of preparing fibrils with known final fibril concentrations and length distribution is essential.

In general, the formation of fibrils occurs in narrow pH and ionic strength ranges. It can be induced by increasing the temperature of the protein solution, to a temperature close to the protein denaturation temperature (*7*−*9*), and/or adding cosolvents such as alcohols, salts, or metal ions, which destabilize the native conformation of the protein (*10*). The formation of fibrils from protein solutions can be characterized by a lag time, a nucleation event, and finally a period of growth (*11*). In the case of HEWL, several methods to prepare amyloid fibrils have been reported. The exact mechanism of HEWL amyloid fibril formation does not have a specific path but depends on the conditions inducing fibril formation (*1*−*4, 12, 13*).

When intact HEWL is introduced to a very concentrated ethanol environment (90% v/v), the process of amyloid formation has several steps. First, the helical content increases, leading to a perturbation of the tertiary structure. Then, the helical structures of HEWL are partly destroyed due to the highly concentrated ethanol solution, which is followed by the association into amyloid fibrils (*2*). In these conditions, fibrils have an average diameter of 7 nm and are as long as 100−200 nm. Cao et al. (*3*) have also used concentrated ethanol (90% v/v) to convert fully reduced HEWL to amyloid fibrils at low pH (pH 4−5), and the fibrillogenesis showed obvious differences. The flexibility of the peptide chain of the fully reduced HEWL (without the four disulfide bond constraint) allowed it to explore much greater conformational space than the disulfide-intact HEWL (*3*). Consequently, the fibrils formed have a diameter of 2−5 nm and are as long as 1−2 μm, much thinner and longer than those formed from the native HEWL (*3*).

Besides alcohols, adding other denaturants, such as 3−4 M guanidine hydrochloride (*13*), or 30% 2,2,2-trifluoroethanol at, respectively, 37 and 65 °C, can produce fibrils (*1*). Also, sonication was found to form amyloid-like aggregates from LSZ due to protein destabilization (*14*). Nevertheless, the most commonly used fibril formation conditions for HEWL (or its peptides) involve low pH (1.6−2.0) and elevated temperatures (57−100 °C) (*1, 4, 12, 15*).

One of the first studies of the formation of HEWL amyloid fibrils by heat treatment was that published by Krebs et al. in 2000 (*1*). In that study, fibrils were formed from a 1 mM HEWL solution at pH 2 by incubation at 37 °C, after rapid heating to 100 °C and freezing in liquid nitrogen. Besides from native proteins, fibril formation from peptides (in aqueous solutions at pH 2 and 4) was also investigated. The authors found that

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fibril formation of HEWL led to amyloid fibrils containing full-length LSZ (when using intact protein) or fragments (when using peptides). The fibrils from either intact HEWL or peptides are, in general, long (>1 μm) and semiflexible (the persistence length is of the same order of magnitude as the contour length). Those from intact protein appear to be a bit thinner [7.4 ($\pm$0.5) nm] than those from peptides [10 ($\pm$3) nm] (*1*).

The effect of thermal conditions on fibrilar aggregation was extensively studied by Arnaudov et al. (*4*). These authors explored the effects of increasing the pH (from 2 to 4) and temperature (from 57 to 80 °C) and found that the optimal conditions for HEWL fibril formation are at pH 2 and 57 °C. At these conditions, the fibrils obtained are semiflexible and long (about 5 μm), and the longest fibrils observed were about 16 μm. Their predominant thickness is about 4 nm, and they appear to be composed of stiff rod-like subunits with lengths of 124 ($\pm$9) and 157 ($\pm$11) nm (*4*). These fibrils seem to consist mostly of full-length HEWL, although some fragments—products of hydrolysis at pH 2 and 57 °C—are probably incorporated into fibrils as well (*4*). This morphology is close to that found by Cao et al. (*3*). The HEWL fibrils formed at pH 3 and 57 °C are also very long (around 5 μm), with a thickness similar to those obtained at pH 2, but they seem more flexible, and no rod-like subunits were observed (*4*). The fibrils formed at 80 °C were reported to be very long, thin, and semiflexible as well (*4*).

In a recent study (*12*), a mechanism of HEWL amyloid fibril formation at low pH and elevated temperature (pH 1.6, 65 °C) was proposed, which incorporates steps of hydrolysis, fragmentation, assembly, and conversion into amyloid fibrils. This model suggests that the formation of HEWL amyloid fibrils is a multiorder process that is highly complex due to a multitude of interactions and the involvement of quaternary intermediates. First, HEWL unfolds, and then partial acid hydrolysis produces a large amount of nicked HEWL and fragments. After the nicked full-length proteins are incorporated into amyloid fibrils, they are further degraded and trimmed to a core composition—a process referred to as "fibril shaving" (*12*). In this study, it is verified that at pH 1.6 and 65 °C, HEWL fragments completely, either before or after incorporation into amyloid fibrils (*12*). Thus, the morphology of HEWL fibrils formed from different conditions and initial states of HEWL (native or reduced) is diverse. Even a small change in the inducing conditions produces fibrils that vary from short to long and from stiff to flexible.

Although several studies have commented that stirring, shaking, or applying mechanical agitation or shear flow influences the rate of fibril formation (*5, 16, 17*), effects of flow on HEWL amyloid fibril formation have remained unexamined. These effects have been studied extensively for β-lactoglobulin by Akkermans et al. (*5, 16, 17*) and Hill et al. (*17*). Akkermans et al. (*5, 16*) have studied the effects of shear flow on the formation of β-lactoglobulin amyloid fibrils in heating conditions. Samples (0.5 wt %, pH 2) were heated at 80 °C with pulsed and continuous shear treatment at a shear rate of 200 s^{-1} (*5*). Results show that shear flow significantly enhances the formation of β-lactoglobulin fibrils. However, there is no difference in the final fibril concentrations between applying continuous shear flow, giving short shear pulses every hour, or giving only one short shear pulse at the start of the heating process. In other words, the onset of the shear flow is the key parameter for enhancing the fibril growth (*5*). The results of samples subjected to different continuous shear treatment with various shear rates, using a shear device with a Couette geometry (pH 2, 90 °C, shear rates varied from 0 to 673 s^{-1}, protein concentrations ranged from 0.5 to 5.2 wt %), showed that the use of shear flow results in a higher amount of fibrils (*16*). However, this effect started only around a protein concentration of 3%. The total length concentration increased as a function of shear rate up to a shear rate of 337 s^{-1}. Above that shear rate, it decreases with increasing shear rate. The length of the fibrils obtained can be influenced only to a minor extent, and it appears that shear flow results in slightly shorter fibrils (*16*).

In this paper, we present the effects of simple shear flow (Couette flow) and turbulent flow during heating on the length distribution and the conversion of HEWL fibrils. We will show that the length of the HEWL fibrils, as well as the amount of fibrils obtained, is strongly affected by flow. The effect of flow on fibril formation is much more significant than in the case of β-lactoglobulin.

MATERIALS AND METHODS

Materials. HEWL was obtained from Sigma-Aldrich Co., St. Louis, MO (3× crystallized, lyophilized powder, product L6876). All other chemicals used were of analytical grade, unless stated otherwise. All solutions were prepared with Millipore water (Millipore Corp., Billerica, MA). Protein solutions contained 200 ppm of NaN_3 to prevent bacterial growth. Samples were diluted to the desired concentration (if applicable) with the same buffer or solution used for the primary solutions.

Preparing HEWL Solutions. Dried powder of HEWL was dissolved in 10 mM HCl (pH 2) and extensively dialyzed against 10 mM HCl (pH 2) at 4 °C to remove dissolved salts present in HEWL (around 5 wt %). The dialyzed protein solution was filtered using a 0.2 μm pore filter size (FP 30/0.2 CA-S, Schleicher & Schuell, Germany), and its concentration was determined by absorbance measurement using a UV spectrometer (Cary 50 Bio, Varian, Inc., Palo Alto, CA) and a calibration curve of known HEWL concentrations at 280 nm. The protein stock solution was diluted with 10 mM HCl to the desired concentrations. The pH of protein solutions was adjusted using a 6 M HCl solution.

Preparing HEWL Fibrils. Arnaudov et al. (*4*) reported that the optimal conditions for fibril formation at low pH were at pH 2 and 57 °C. On the basis of these results, our HEWL solutions of 2 wt % were heated at 57 °C with and without applying continuous turbulent or shear flow. After heating, the sample was immediately quenched by cooling on ice and subsequently stored at 4 °C for further investigations. Crossed polarizers were used to observe whether the fibril solutions were optically isotropic or birefringent at rest.

(a) *Steady Shear Experiments.* A shearing device with a titanium Couette geometry was used to perform simultaneous heating and shearing with minimum water evaporation (*16*). The diameter and length of the inner cylinder were 40 and 145.5 mm, accordingly. The diameter of the outer cylinder was 42 mm, and the length was 148.5 mm. The top and bottom of this device were designed as a cone-and-plate geometry with an angle of 2.8°, which made the shear rate between the cone and plate comparable to that between the two cylinders. The sample volume of this shearing device was 18 mL. During the shearing experiment, the inner cylinder was rotated by a mechanical stirrer (type 2041, Heidolph Instruments, Schwabach, Germany), and the cell was heated in a water bath (Tamson 2500, Labovisco B.V., Zoetermeer, The Netherlands) for 24 h (*16*).

(b) *Turbulent Flow Experiments.* Stirred samples were heated in a heating plate (RT15, IKA Werke, Germany) using magnetic stirring bars (20 mm × 6 mm, VWR International, West Chester, PA). Bottles with an inner diameter of 24 mm and containing about 18 mL of sample were used in this experiment. Stirring rates of approximately 290 and 550 rpm were applied. Samples of each stirring rate were investigated at various heating times.

Methods. *Centrifugal Filtration Method.* This method was carried out as described in a study of Bolder et al. (*18*). Fibril solutions were diluted to 0.1 wt % with a 10 mM HCl solution (pH 2). Diluted samples (3.5 mL) were then brought into centrifugal filter devices (Amicon Ultra-4, Millipore Corp.) with 100,000 nominal molecular weight limit and filtered by centrifuging at 5000*g* for 20 min (Avanti J-26 XP,

Beckman Coulter, Inc., Fullerton, CA). The obtained filtrates (containing nonaggregated proteins) were transferred, and the centrifuge tubes were washed and dried for the next step. The retentates in the filter units were washed twice with 10 mM HCl solution (pH 2) by gentle mixing and then centrifuged at the same conditions to remove any nonaggregated materials left after the previous filtration step. The protein concentrations in various fractions were determined using a UV spectrometer (Cary 50 Bio, Varian, Inc., Palo Alto, CA) at a wavelength of 280 nm. The experiment was performed in duplicate.

Gel Electrophoresis (SDS-PAGE). Polyacrylamide gel electrophoresis (PAGE) was performed using a NuPAGE Electrophoresis System (Invitrogen Corp., Carlsbad, CA). Samples (nonreduced) were run on 4−12% Bis-Tris gels (1.0 mm × 10 well) with MES SDS running buffer and stained with Simply Blue SafeStain (Invitrogen Corp.). This method was performed on HEWL fibril solutions, filtrates, and retentates from the centrifugal filtration method to examine the presence of proteins, their fragments, and aggregates of different molecular weights.

Transmission Electron Microscopy (TEM). HEWL fibril solutions were examined using TEM by negative staining. Samples were first diluted (the dilution factor varied from 2000 to 4000 times depending on the fibril concentration and fibril length). Then, a drop of diluted sample was deposited onto a 5 nm thick carbon support film on a copper grid (400 mesh). Filter paper was used to remove the excess of sample after 15 s, before adding a droplet of staining solution (2% uranyl acetate, Sigma-Aldrich, Steinheim, Germany). After another 15 s, the excess was removed and the sample was left to air-dry. The micrographs were taken using a Philips CM 12 electron microscope operating at 80 kV (Eindhoven, The Netherlands) (*16, 18*). From the TEM pictures, fibrils were counted and their length was measured to produce the size distribution of the fibrils. To get reliable statistics, between 240 and 380 fibrils were analyzed for each sample.

Rheo-optical Measurements. Birefringence measurements of HEWL fibril solutions were performed on a strain-controlled ARES rheometer (Rheometrics Scientific, Inc., Piscataway, NJ) equipped with a modified optical analyzer module II (OAM II). Glass parallel plates with a diameter of 38 mm and a gap of 1 mm were used. The wavelength of the laser beam was 670 nm. In this setup, the apparatus was capable of measuring birefringence in the range from 10^{-9} to 8×10^{-6} at a sampling frequency of 24 Hz. The measurements were performed in a temperature-controlled room at 20 °C (*16, 19, 20*).

Two types of test were performed: steady shear and cessation of flow experiments. In the steady shear tests a shear rate of 200 s^{-1} was used to completely align the fibrils (a shear sweep was performed to confirm that this shear rate was indeed sufficient to completely align the fibrils). The birefringence measured at total alignment is a measure for the total length concentration of the fibrils (*21−23*). By combining the results of these tests with the data for the conversions from the centrifugal centrifugation experiments, a fast assay can be constructed for the conversion into fibrils (*19*).

In the cessation of flow tests, the measured decay of birefringence curves was used to determine the length distribution of the fibrils, using a method developed by Rogers et al. (*21*). Conditions were identical to those used by Rogers et al. (*21*).

RESULTS AND DISCUSSIONS

Fibrils were produced in the steady shear cells at shear rates ranging from 71 to 436 s^{-1} and turbulent flows at two flow rates (290 and 550 rpm). The samples were put between crossed polarizers, and they showed permanent birefringence (see **Figure 1**), indicating that a significant amount of fibrils is present in all samples (*19*). We see that at concentrations as low as 2 wt % the systems are no longer isotropic, and liquid crystal-like domains have formed in the solutions.

Conversion Experiments. In these experiments the conversion is defined as the weight percentage of aggregated protein material. The conversion of HEWL protein at pH 2 as a function of shear rate (varying between 71 and 436 s^{-1}) was determined for protein solutions of 2 wt %, heated at 57 °C for 24 h, using the centrifugal filtration method. The results of the centrifugal

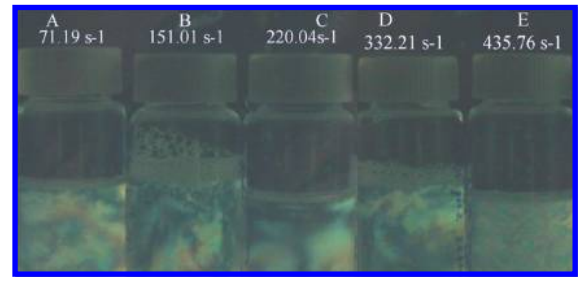

Figure 1. Samples produced by heating in steady shear cells at various shear rates: (**A**) 71 s^{-1}; (**B**) 151 s^{-1}; (**C**) 220 s^{-1}; (**D**) 332 s^{-1}; (**E**) 436 s^{-1} placed between crossed polarizers.

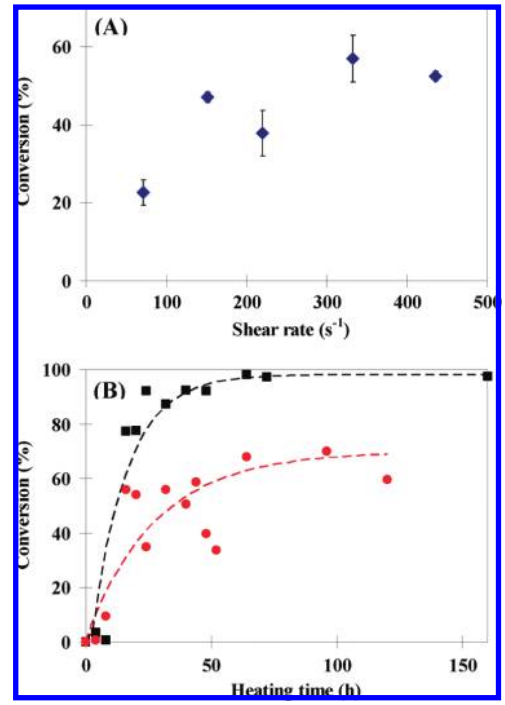

Figure 2. (**A**) Conversion as a function of shear rate for 2 wt % HEWL heated at 57 °C for 24 h. The error bars indicate deviation from mean values of duplicate samples. (**B**) Conversion as a function of heating time for 2 wt % HEWL heated at 57 °C at two stirring rates: (■) 550 rpm; (●) 290 rpm.

filtration experiments of sheared samples are presented in **Figure 2A** and show a significant increase in conversion with increasing shear rate, up to shear rates of 200 s^{-1}. At higher shear rates no further increase in conversion is observed.

For most shear rates, the conversions of duplicate samples were comparable, except for samples prepared at shear rates of 220 and 332 s^{-1}. These show a slightly higher variation most likely due to minor deviations from the applied shear rate that occurred in the early stages of these experiments.

A clear trend can be seen in all of these samples: an increase of shear rate leads to an increase in conversion. Even at the lowest shear rate, >20% of the protein was already aggregated into fibrils after 1 day of shearing, compared to a lag time of 2 days in which no conversion can be detected when HEWL solutions were heated at rest (*4*). This shows the significant effect of shearing on the HEWL fibril aggregation.

A similar observation on β-lactoglobulin was reported in a study of Akkermans et al. (*5*). However, in the case of β-lactoglobulin, the effect of shear flow on fibril concentration at low protein concentrations (0.5−2% wt) was not as pronounced as it was in our HEWL samples (for β-lactoglobulin

samples fibril length is reduced by at most a factor of 2 when shear is applied) (*16*). This comparison confirms that although amyloid formation appears to be a generic property of many globular proteins (or their peptide fragments), the mechanism of self-assembly varies and seems to be specific for each one (*7*).

For the stirred samples (turbulent flow), the conversion as a function of time for two different stirring settings is plotted in **Figure 2B**. At a speed of 550 rpm, HEWL fibril solutions reached a conversion of around 90% after 2 days. A conversion of about 60% was found over the same period of time for samples stirred at 290 rpm. These conversions are significantly higher compared to those for samples at the same conditions (concentration, pH, and heating temperature), and heated at rest, reported by Arnaudov et al. (*4*). In our own samples some fibrils were seen on TEM micrographs of samples heated at rest after 24 h of heating (micrographs not shown), but the conversions could not be determined due to the low fibril concentrations. The conversions after 24 h of heating in turbulent flow were in the same range as those of sheared samples (between 30 and 60%). Therefore, both types of flow enhanced the formation of HEWL fibrils significantly.

After centrifugal filtration, the filtrates and retentates were examined using TEM and SDS-PAGE methods. When the filtrates were studied with TEM, no aggregates were observed (micrographs not shown), showing that the loss of fibrils was negligible. In addition, the SDS-PAGE tests (see **Figure 3A**) of retentates and filtrates showed that the bands of HEWL monomers became less visible after each of the washing steps and entirely disappeared from both retentates and filtrates after the last centrifugation step (for more electrophoretograms, see Supporting Information). That means that the washing steps were adequately separating aggregates from nonaggregated material. The presence of high molecular mass bands in the wells of retentate lines (see **Figure 3B**) indicated the presence of aggregates of molecular mass larger than 200 kDa (marker Mark12, Invitrogen Corp., has a molecular mass range from 2.5 to 200 kDa in the conditions applied). However, no other intermediate aggregates were obvious on the gels. The same observation was reported by Mishra et al. of samples at pH 1.6 incubated for >40 h at 65 °C (*12*). The high molecular weight material in the final retentate was investigated using TEM (see **Figure 3C**). This image clearly shows that this material consists of long fibrils.

Length Distributions. In the analyses of TEM micrographs, images of fibrils of sheared samples were captured randomly on the grids, and their contour lengths were measured to construct a length distribution. Sample sizes varied from 240 to 380 fibrils. Only fibrils that could be clearly distinguished and had both ends on the image were analyzed. The number of fibrils of length L, N, was weighed by $1/(1 - L/D)$, the probability of finding a fibril of length L in a frame with width D (*21*). The results are presented in **Figure 4A**.

The maximum in the curves shifted from right to left (from long to shorter fibrils) as a result of increasing shear rates (**Figure 4A**). The predominant lengths of HEWL fibril solutions sheared at 71, 220, and 436 s^{-1} are approximately 800, 1300, and 1500 nm, respectively. Thus, the increasing shear rate not only induces an increase in the fibril formation rate but also shortens the fibrils formed. A possible explanation for these observations could be that shearing ruptures the fibrils during formation, producing two or more active fragments, which each may grow by addition of HEWL monomers (or peptide fragments). Consequently, more and shorter fibrils are formed.

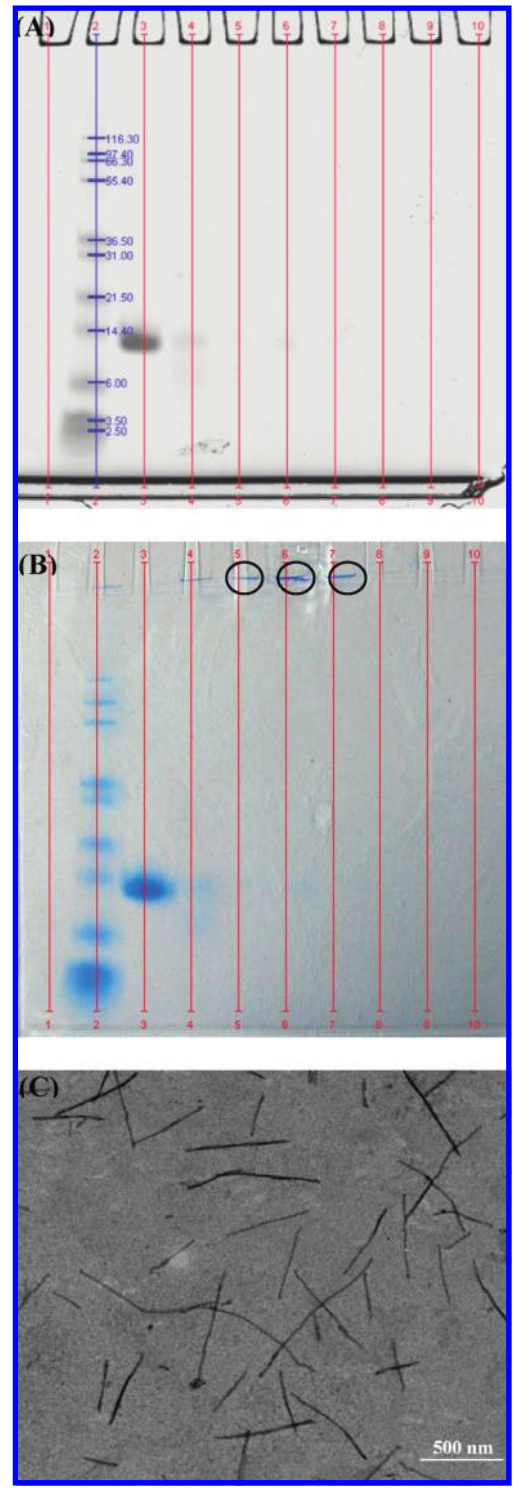

Figure 3. SDS-PAGE electrophoretograms of filtrates and retentates of samples heated at 290 rpm for 96 h: (**A**) scanned image using the software Quality One (Bio-Rad Laboratories GmbH, Munich, Germany); (**B**) digital image [lane 1, blank; lane 2, marker Mark12 (Invitrogen Corp., Carlsbad CA); lane 3, purified LSZ (after dialysis) as starting material; lane 4, LSZ fibril obtained after heating at 57 °C for 96 h with a stirring rate of 290 rpm; lanes 5−10, respectively, retentates 1, 2, and 3 and filtrates 1, 2, and 3 (obtained after the first, second, and last centrifugation steps); the presence of a high molecular mass band in the wells of retentate lanes was marked with an ellipse]; (**C**) TEM micrograph of retentate 3 (obtained after the last centrifugation step) (scale bar represents 500 nm).

For stirred samples, the TEM analyses were carried out similarly to the sheared samples, but the sample sizes were

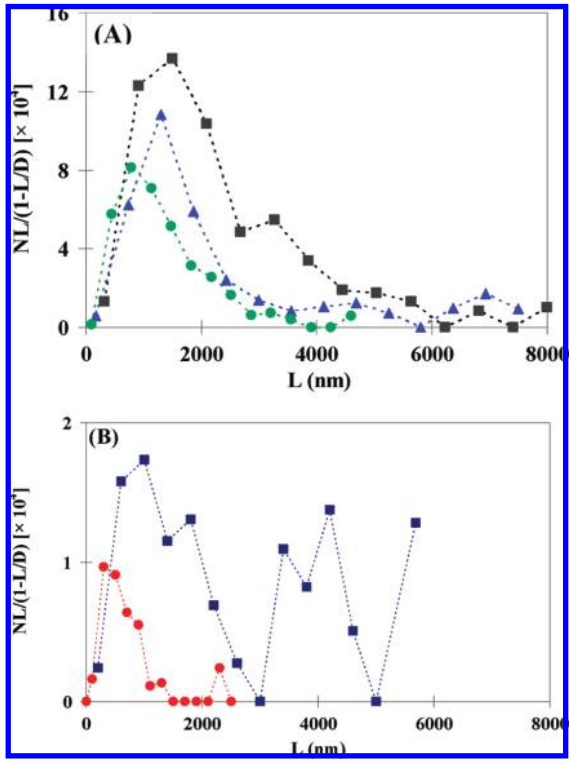

Figure 4. (**A**) Weighted length distribution curves of samples prepared at different shear rates (■, 71.19 s^{-1}; ▲, 220.04 s^{-1}; ●, 435.76 s^{-1}); (**B**) weighted length distribution curves of samples heated for 48 h at different stirring rates (■, 290 rpm; ●, 550 rpm).

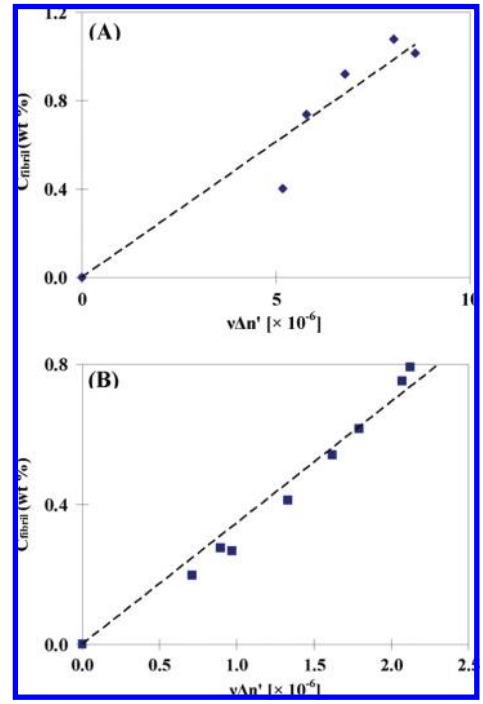

Figure 5. Correlation between the measured steady shear birefringence corrected with the dilution factor, $\nu\Delta n'$, and the average fibril concentration based on the conversion of HEWL as determined using the centrifugal filtration method, C_{fibril}. (**A**) samples produced in steady shear flow; (**B**) samples produced in turbulent flow at 290 rpm.

somewhat smaller (around 100 fibrils per sample were measured). Samples of the same stirring rates but at different heating times were investigated, but no obvious difference in size distribution was observed. Therefore, heating time did not have an effect on the size of fibrils formed.

The length distributions obtained from TEM of samples in turbulent flow were much wider than those of samples produced in steady shear flows (**Figure 4**). At lower stirring rates, polydispersity is more pronounced than at higher stirring rates. This is most likely a result of the inhomogeneity of the flow patterns. At low stirring rates only the portion of the fluid close to the magnetic stirrer bar is exposed to significant shear and extensional rates, whereas the fluid close to the top of the container is hardly sheared at all. Consequently, the sizes of fibrils varied significantly, as can be seen from the presence of a second peak in samples at 290 rpm (**Figure 4B**). Inhomogeneous flow apparently leads to the formation of a mixture of very long and short fibrils. Note that at 290 rpm the second peak in **Figure 4B** is rather pronounced. In this distribution each fraction is weighted by a factor $1/(1 - L/D)$, where D is the width of the frame of the TEM micrograph. This implies that longer fibrils contribute more to the total length distribution. **Figure 4B** shows that fibrils formed at higher stirring rates were shorter than those at lower stirring rate. The peaks in the distribution are at approximately 300 and 650 nm for samples stirred at 550 and 290 rpm, respectively, whereas for samples produced in steady shear the peaks were at lengths above 1 μm.

Birefringence of Fibril Solutions. *Birefringence and Fibril Concentration.* HEWL fibril solutions with known conversions, determined with the centrifugal filtration method, were subjected to steady shear flow at a shear rate of 200 s^{-1}, for 30 s, in both clockwise and counterclockwise direction. This shear rate was sufficient to induce maximum alignment of the fibrils. Samples were diluted 10 times prior to measurement of the flow birefringence $\Delta n'$. This value is related to the length and concentration of fibrils by the equation (*21*)

$$\nu\Delta n' = M\int c(L)L\,\mathrm{d}L \qquad (1)$$

where ν is the dilution factor, M is a constant (*21*), and $c(L)$ is the concentration of fibrils with a length between L and $(L + \mathrm{d}L)$.

Plotting the birefringence versus the fibril concentration (**Figure 5A**) we obtain a linear relationship, which is basically the inverse of eq 1. The equation of the trend line is given by $C_{\text{fibril}} = K_1\nu\Delta n'$, with a slope $K_1 = 1.22 \times 10^5$. From this value for K_1 a value for the constant M can be calculated, using (*21*)

$$M = \frac{\alpha M_{\text{mono}}}{10K_1N_{\text{Av}}} \qquad (2)$$

where M_{mono} is the molecular mass of the protein monomers (14.4 kDa), α is the fibril line density (monomers per unit length), and N_{Av} is Avogadro's number (6.022×10^{23} mol^{-1}). The line density, determined by neutron scattering measurements, was approximately equal to the inverse of the monomer diameter (*24, 25*). On the basis of a mean radius of roughly 1.7 nm for the HEWL monomer (*26, 27*), the fibril line density α was chosen to be 0.294 nm^{-1}. Substituting these values in eq 2 resulted in a value for M equal to 5.74×10^{-21} m^2.

In **Figure 5B** we have plotted the concentration of fibrils (determined with the centrifugal filtration methods) versus the birefringence $\nu\Delta n'$, for the samples produced in turbulent flow at 290 rpm.

The slope of the trend line, K_1, was equal to 3.47×10^5, which gives a value for M for these samples equal to 2.03×10^{-21} m^2. This is almost 3 times smaller than the value of M

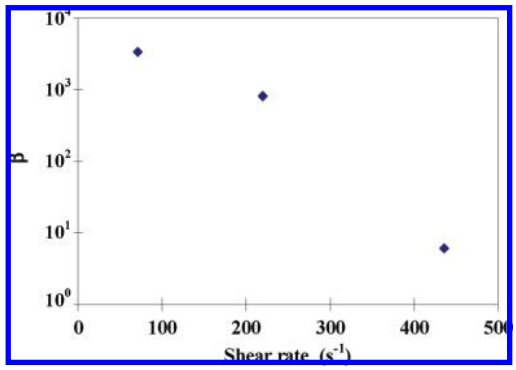

Figure 6. Parameter β as a function of applied shear rate.

for fibrils produced in steady shear flow. A possible explanation for this result could be that turbulent flow has an effect on the fibril shaving process as described by Mishra et al. (*12*), leading to differences in the structure of the fibrils and, therefore, to a different value for the anisotropy in polarization per unit length of the fibrils. However, further research into the actual structure of the fibrils is needed to confirm this hypothesis.

In these birefringence experiments the maximum fibril concentration that could be used was <1 wt %. When the fibril concentration is higher, domains are formed in the sample with permanent birefringence, and the linear relationship in **Figure 5B**, valid only for samples that are isotropic at rest, no longer applies.

Decay Curves and Length Distributions. Rogers et al. (*21*) developed a method to determine the length distribution of rod-like fibril systems from the decay of flow-induced birefringence after cessation of flow. TEM observations showed that the majority of fibrils formed in the conditions investigated are long, thin, and mostly unbranched. That makes HEWL fibril systems suitable for this method, which is based on the Doi−Edwards−Marrucci−Grizutti theory for free, unbranched, nonsticky rods, in the semidilute regime. A shear rate of 5 s^{-1} was applied to all samples. This shear rate ensures that fibrils are sufficiently aligned to accurately study their rotational dynamics, but avoids complete alignment and stretching of the fibrils. At complete alignment the system would be in the dilute regimen, where the DEMG theory is no longer applicable.

The length distribution obtained from the decays was calibrated by comparison with the length distribution from TEM measurements (*21*). The calibration was done by adjusting the value for β—the prefactor in the Doi−Edwards expression for the rotational diffusion coefficient of the fibrils. This prefactor is a measure of the stiffness of the fibrils: it is of order 1 for rod-like fibrils and much higher for semiflexible fibrils (*19*). The adjustment was performed using Matlab software (The MathWorks, Inc., Natick, MA) with an iteration of 100 steps. In **Figure 6** we have plotted the value for β as a function of the applied shear rate. In **Figure 7** we show the size distributions for the same three shear rates.

We clearly see in **Figures 6** and **7** that an increase in shear rate resulted in a shift in the size distribution to smaller fibril lengths and a decrease in β, indicating that fibrils are more rod-like. At low shear rates the fibrils appear to be long and semiflexible, whereas at high shear rates the fibrils appear to be short and rod-like. The effect of shear treatment on the length distribution of HEWL fibrils is much more pronounced than the effect observed for β-lactoglobulin fibrils in about the same shear rate range (*16*). In comparison with

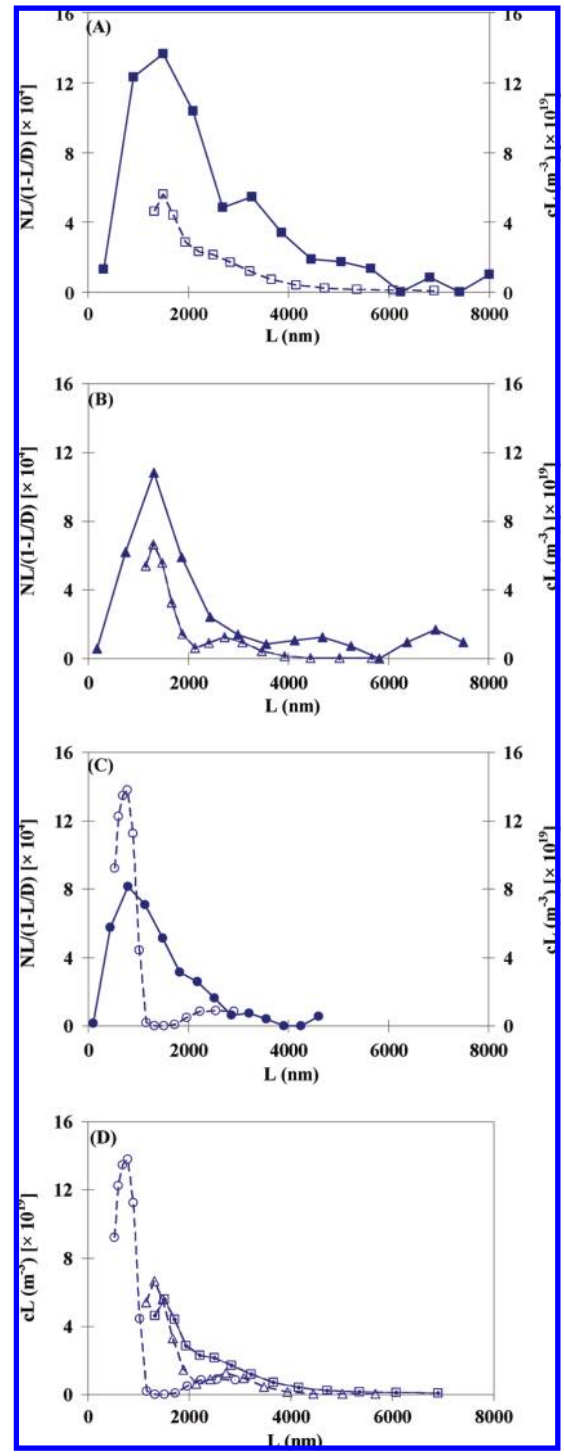

Figure 7. Comparison of the length distributions of samples at various shear rates from fitted birefringence decays (open symbols) and TEM (solid symbols): (**A**) □, ■, 71.19 s^{-1} ($\beta = 3.3 \times 10^3$); (**B**) △, ▲, 220.04 s^{-1} ($\beta = 800$); (**C**) ○, ●, 435.76 s^{-1} ($\beta = 6$). (**D**) Length distributions of samples at various shear rates from fitted birefringence decays (□, 71.19 s^{-1}, $\beta = 3.3 \times 10^3$; △, 220.04 s^{-1}, $\beta = 800$; ○, 435.76 s^{-1}, $\beta = 6$).

HELW fibrils formed at rest, fibrils obtained in shear flow experiments tend to be much shorter and more rod-like (*4*).

In the case of samples stirred at 550 rpm, the decay of birefringence was too fast (even with nondiluted samples) to get a reliable length distribution. The fibrils are too short and the rotational diffusion too fast to determine a size distribution from decay of birefringence measurements.

Therefore, the determination of the length distribution was performed only for samples stirred at 290 rpm.

Fitting the decay of birefringence measurements for the sample produced with turbulent flow at 290 rpm, with the length distributions determined from TEM, gave a value for β of order 10, comparable to that of samples produced in steady shear, at the highest shear rates. This shows that the fibrils have rod-like behavior. This can be explained by the fact that the samples consist predominantly of fibrils in the sub-micrometer range (**Figure 4B**).

Conclusions. From the results of our study, both steady shear and turbulent flows significantly increase the conversion of HEWL proteins into fibrils. The conversion increased with increasing shear or stirring rate. In addition, higher shearing or stirring rates resulted in shorter fibrils that are more rod-like, whereas at rest or at the lowest rates longer semiflexible fibrils were obtained. In simple shear flows length distributions of fibrils were not as wide as those formed in turbulent flows. This is most likely a result of the inhomogeneity of the flow patterns in turbulent flow. The effect of flow on fibril formation from HEWL is much more significant than the effect observed for β-lactoglobulin (*5, 16, 23*), in terms of both conversion and size distributions. The significant effect of flow on the size distribution can be used to control the functionality of the fibrils. A wide range of sizes (from 300 nm to several micrometers) and flexibilities (from rod-like to semiflexible) can be obtained by adjusting the flow rate during the production of the fibrils.

The results from the birefringence measurements show that this technique is an excellent tool to characterize the kinetics of the fibril formation process and the characteristics (length distribution and flexibility) of the fibrils. The values for K_1, M, and β can be used in fast assays for future HEWL fibril formation studies.

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Supporting Information Available: SDS-PAGE electrophoretograms of filtrates and retentates obtained after centrifugation steps. This material is available free of charge via the Internet at http://pubs.acs.org.

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