FOBI (2006) 1: 144–150
DOI 10.1007/s11483-006-9012-5

ORIGINAL RESEARCH

Shear Pulses Nucleate Fibril Aggregation

Cynthia Akkermans · Paul Venema · Salman S. Rogers · Atze Jan van der Goot · Remko M. Boom · Erik van der Linden

Published online: 23 May 2006

Abstract We have studied the effect of shear flow on the formation of amyloid fibrils of the whey protein β-lactoglobulin. β-Lactoglobulin aggregates into long, thin, and semiflexible fibrils upon heating at low pH and low ionic strength. Solutions with a protein concentration of 0.5% (w/w) were used, and the formation of fibrils was quantified with flow-induced birefringence, a proportional measure of the length concentration of the fibrils. From the decay of the birefringence after cessation of the flow, a length distribution could be fitted. Pulsed and continuous shear treatment of the samples resulted in a comparable enhancement of the fibrillar growth as compared to the fibrillar growth under quiescent conditions. This indicates that the onset of shear flow is the key parameter for the enhancement of fibrillar growth and not the continuous shear flow itself. This behavior is comparable to a nucleation-like process, during which preaggregates of the fibrils are induced during the onset of the flow and orthokinetic coagulation is absent. However, a difference was present in the length distribution between the pulsed and continuously sheared samples, which can be explained by the homogenizing effect of shear flow.

C. Akkermans · P. Venema · E. van der Linden (✉)
Food Physics Group,
Wageningen University, P.O. Box 8129,
Wageningen, The Netherlands
e-mail: Erik.VanderLinden@wur.nl

C. Akkermans · A.J. van der Goot · R. M. Boom
Food and Bioprocess Engineering Group,
Wageningen University, P.O. Box 8129,
Wageningen, The Netherlands

S. S. Rogers
Department of Physics,
Cambridge University, Cavendish Laboratory,
Cambridge, CB3 OHE, UK

Keywords Protein · Beta-lactoglobulin · Fibrils · Aggregation · Nucleation · Shear flow

Introduction

The assembly of proteins into amyloid fibrils has received considerable attention in a variety of research fields, from medical[1–3] to materials science.[4,5] Recently, the assembly of proteins into fibrils has gained more attention in the field of food technology, mainly because of the potential utility in modifying the material properties of food products. Examples of food proteins that have shown the ability to aggregate into fibrils are β-lactoglobulin, bovine serum albumin, lysozyme, and ovalbumin.[6–10] An interesting protein on which to study the effect of process parameters on the fibril formation is β-lactoglobulin because the fibrils formed by this protein have a large aspect ratio, are polydispers, and are semiflexible. This enables us to picture them as rod-like particles. For these rod-like systems, a method has been developed recently, which links the decay of birefringence directly to the length distribution.[11]

The most common treatment of β-lactoglobulin solutions to induce the formation of fibrils consists of prolonged heating (6–24 h) at 80°C at pH 2 and low ionic strength.[6,11–14] The average length of the fibrils depends on the conditions used (e.g., heating time, ionic strength, pH, concentration), but is typically found to be in the range of 1–8 μm.[6,11–14] The diameter of the fibrils is around 4 nm, which implies a thickness of 1 or 2 monomers.[13] The molecular mechanism of the formation of β-lactoglobulin fibrils is not yet clear. During the fibril formation, an increased amount of intermolecular β-sheets was detected with Fourier transform infrared spectroscopy; these β-sheets may bind the monomers together in the fibril.[14,15] Dynamic light scattering showed that after short heating times,

the fibrils disintegrate during cooling, but after longer heating times, the fibrils do not disintegrate upon cooling.[13]

For the industrial application of fibrils in food products, an acceleration of the fibril formation would be advantageous. Enhanced formation of different protein fibril systems by addition of preformed fibrils (seeding) has previously been established.[16–18] Another possibility is inducing a mechanical disturbance in the system. In insulin solutions, continuous agitation was shown to enhance the fibril formation.[17] Unfortunately, these experiments were not performed in a well-defined flow field. Stathopulos et al.[18] found that sonication of a diverse group of proteins resulted in the formation of fibrils. However, these sonication experiments are characterized by an approximately 10^6-fold higher energy input than the energy input of the shear treatment used for our experiments.

To analyze the fibrils, transmission electron microscopy or atomic force microscopy can be used to determine the length distribution of the fibrils,[6,13,14] but these techniques are very laborious and often give only semiquantitative information. Other methods that can be used to measure the fibril concentration include the binding of fluorescent dyes (e.g., Congo Red[19,20] and thioflavin T[16,17]), but these methods give no information on the fibril length distribution. During this study, flow-induced birefringence was used to analyze the fibril solution. The major advantage of using flow-induced birefringence is that both the concentration and the length distribution of the fibrils can be measured quantitatively in situ.[11] Apart from flow-induced birefringence, static light scattering was used to detect the presence of aggregates (in situ) in the early stages of the aggregation process.

In this paper, we report on the effect of applying the mechanical disturbance of simple shear flow to the formation of β-lactoglobulin fibrils. We found that the onset of the shear flow is the key parameter for the enhancement of fibrillar growth, and we propose that this is caused by a nucleation-growth mechanism.

Materials and methods

Preparation of β-lactoglobulin stock solution

β-Lactoglobulin (18.3 kDa) was obtained from SIGMA and contained the genetic variants A and B. The stock solution of the protein was made by dissolving the protein in a HCl solution of pH 2. To remove traces of electrolytes, the solution was dialyzed (dialysis tube of 12–14 kDa from Visking) against an HCl solution of pH 2 until the pH and ionic strength of the protein solution were equal to the solvent. To remove traces of undissolved protein, the solution was centrifuged at 22600 × *g* for 30 min and, subsequently, filtered through a protein filter (FP 030/0.45 μm, Schleicher & Schuell). The concentration of the β-lactoglobulin stock solution was determined with a UV spectrophotometer at a wavelength of 278 nm, using a calibration curve of known β-lactoglobulin concentrations.

Sample treatment

The β-lactoglobulin concentration of all samples in this study was 0.5% (w/w). This concentration is far below the minimum gelation concentration (under no shear condition) of 2.3% at an ionic strength of 0.01 M,[6] and this concentration resulted in isotropic fibril solutions. During the experiments, the solutions were kept at pH 2, and no electrolytes were added.

Table 1 shows an overview of the treatment of different samples (A–I). As a pretreatment, samples were heated at 90°C in glass tubes, followed by cooling at room temperature for 1 h. The heating times of the samples were 0, 2, or 10 h. During the pretreatment, no shear treatment was applied. After the pretreatment, the following different treatments were applied: no shear, continuous shear (at a shear rate of 200 s^{-1}), or short shear pulses of 30 s at a shear rate of 200 s^{-1}. A shear rate of 200 s^{-1} was selected because we expected the effect of shear flow on the fibril formation to be more pronounced at high shear rates. This shear rate is close to the highest shear rate possible without the risk of expelling the sample from the Couette geometry.

Measurements of the nonsheared samples were performed on different samples. For measuring the flow-induced birefringence, shear flow needs to be applied, and after the measurement, the sample cannot be regarded as a nonsheared sample anymore. Therefore, the different nonsheared samples are numbered, and each number refers to a different sample.

Flow-induced birefringence

The samples were subjected to shear flow in a rheometer (ARES, Rheometric Scientific Ltd.) with Couette geometry (rotating cup with a diameter of 33.8 mm and a static bob with a diameter of 30.0 mm) at a shear rate of 200 s^{-1} for 30 s. During the shear treatment, the flow-induced birefringence was measured. A laser beam of wavelength 670 nm passed vertically through the gap between the cup and bob, and the birefringence was measured with a modified optical analysis module (OAM; Klein et al., unpub-

Table 1 Overview of sample treatment

Sample	Heating time of pretreatment (h)[a]	Treatment		Measurement time (hours after pretreatment)[b]
		Type	Time (hours after pretreatment)[b]	
A	0	No shear		0
B1	2	No shear		0
B2	2	No shear		5
B3	2	No shear		22.5
C1	10	No shear		0
C2	10	No shear		5
D[c]	0	Continuous shear[d]	5	0–5
E[c]	2	Continuous shear[d]	5	0–5
F[c]	10	Continuous shear[d]	5	0–5
G	2	Shear pulses[e]	0, 1, 2, 3, 4	0, 1, 2 , 3, 4, 5
H	2	Shear pulses[e]	0	0, 5
I	2	Shear pulses[e]	5	5, 25

[a] The pretreatment consisted of heating, followed by 1-h cooling at room temperature. During the pretreatment, no shear treatment was applied.
[b] $t = 0$ refers to the time at which the shear treatment started.
[c] Continuous measurement.
[d] The shear rate of continuous shear treatment was 200 s^{-1}.
[e] Pulsed shear treatment comprised of a shear pulse of 30 s at a shear rate of 200 s^{-1}. The time refers to the time at which the shear pulse was given.

lished). The rheo-optical analysis was performed in a temperature-controlled room at 20°C.

At complete alignment of the fibrils, the flow-induced birefringence (Δn) is proportional to the total length concentration of the fibrils:[11]

$$\Delta n = M \cdot \int c_L \cdot dL \tag{1}$$

In Eq. (1), c_L is the contribution to the total length per unit volume in the system as a result of the fibrils with a length between L and $L + dL$. The constant M is the contribution to the birefringence per unit length concentration and was determined for β-lactoglobulin fibrils by Rogers et al.[11]

The applied shear rate was 200 s^{-1} because this was the highest rate experimentally possible. At a higher shear rate, part of the sample would be expelled from the measuring cup. During the steady shear of 30 s, a constant birefringence was measured for all samples, indicating that the amount of fibrils did not change during the measurement. Furthermore, Rogers et al.[11] also performed birefringence measurements on β-lactoglobulin fibrils, and they showed that the fibrils did not break up during the shear treatment of the measurement up to shear rates of 200 s^{-1}.

Length distribution of the fibrils

To determine the length distribution of the fibrils, the samples were subjected to steady shear flow at a shear rate of 200 s^{-1} for 30 s. After cessation of the flow, the decay curve of the birefringence was measured. Rogers et al.[11] developed a method to deduce the length distribution of β-lactoglobulin fibrils from this decay curve, based on the Doi-Edwards-Marruci-Grizzuti (DEMG) theory.[21–23] A simplified version of this method was used: each decay curve was fitted by assuming a Gaussian distribution function for the length distribution. The mean and variance of this Gaussian were adjusted to provide the closest fit of the decay curve via the DEMG theory.

Static light scattering

Static light scattering was measured with a Multi Angle DAWN®EOS™ laser photometer from Wyatt Technology. At room temperature, a β-lactoglobulin solution was injected into the flow cell. The sample was heated to 90°C at a rate of 1°C/min. When this temperature was established, the solution was kept at this temperature for 2 h. After the heat treatment, the solution was cooled to 20°C (1°C/min). Because no angle dependency was found, the scattered intensity at an angle of 90° was used.

Results

Effect of pretreatment (in absence of shear flow)

In this section, the effect of the pretreatment (heating and subsequent cooling) on the fibril formation

without applying shear treatment is described. These experiments will serve as a reference relative to which the different shear treatments are compared. Using static light scattering, the presence of aggregates was studied during a 2-h heat treatment and 1-h cooling.

In Figure 1, the scattering intensities (at an angle of 90°) are shown for duplicated experiments at three different moments: before heating, after heating for 2 h at 90°C, and after cooling. Before heating, the scattered intensities corresponded to the intensity of native β-lactoglobulin molecules. Immediately after heating for 2 h, the scattered intensities were higher than the intensities before heating, implying that aggregates had formed during heating. After cooling, the scattered intensities decreased, meaning that aggregates disintegrated during cooling. The scattered intensities did not decrease to the same level as before heating, suggesting that a part of the aggregates formed during heating were still present after cooling. The size of the aggregates was estimated to be in the range of nanometers because no angle dependence was found for the scattered intensity. Because a low protein concentration was used and initial times of aggregation were studied, the scattered intensity resulting from a change in second virial coefficient was assumed to be low compared to the scattered intensity of the aggregates. Arnaudov and de Vries[24] have used light scattering in a similar way for β-lactoglobulin aggregates at low protein concentration.

The presence of fibrils after heating was detected using flow birefringence at a shear rate of 200 s^{-1}. Different heating times (0, 2, and 10 h) were used, and the birefringence was measured at lag times that were comparable to those used for the shear treatments. Figure 2 shows the birefringence of the samples after different lag times (samples A, B1, B2, B3, C1, and C2; Table 1). The numbers after B and C indicate that different nonsheared samples were used for each measurement. For measuring the flow-induced birefringence, shear flow needed to be applied, meaning that for each measurement time, a "fresh" nonsheared sample was needed.

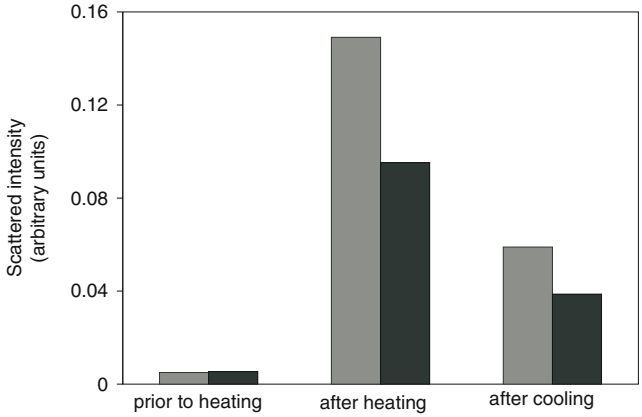

Fig. 1. Scattered intensity of lactoglobulin solutions (0.5%) before heating, after heating for 2 h at 90°C, and after cooling to 20°C (performed two times).

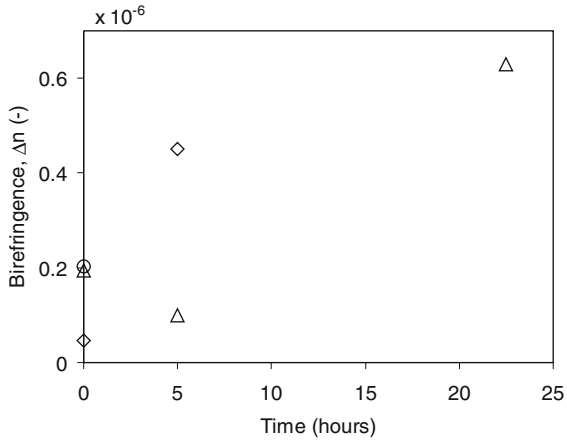

Fig. 2. Birefringence signals at different lag times after various heating times (samples A, B1, B2, B3, C1, and C2; Table 1): (○) 0 h, (△) 2 h, (◇) 10 h.

At $t = 0$ h (immediately after heating and cooling), a negligible flow birefringence was observed for all three heating times. After a lag time of 5 h, the sample heated for 2 h (B2) did not show an increase of flow birefringence, but the sample heated for 10 h (C2) gave a significant flow birefringence; an appreciable yield of fibrils was achieved after 10 h of heating and a 5-h lag time. After a 22.5-h lag time, the sample that was heated for 2 h (C3) also showed an increase of birefringence, meaning that the concentration of fibrils was significantly higher after a 22.5-h lag time than after a 5-h lag time.

Effect of continuous shear treatment

The effect of continuous shear flow on the formation of the β-lactoglobulin fibrils was determined by subjecting heated samples to continuous shear flow for 5 h (samples D, E, and F; Table 1). Shear treatment was applied at room temperature. Figure 3 shows the increase of birefringence during continuous shear treatment after heating for 2 and 10 h. The sample heated for 0 h (sample D) did not show any increase of birefringence during continuous shear treatment for 5 h and is not shown in Figure 3. Heating for 2 h followed by continuous shear treatment for 5 h (sample E) was performed three times to test the experimental variability, which turned out to be quite modest. The trends observed under different treatment conditions were much larger than this variability.

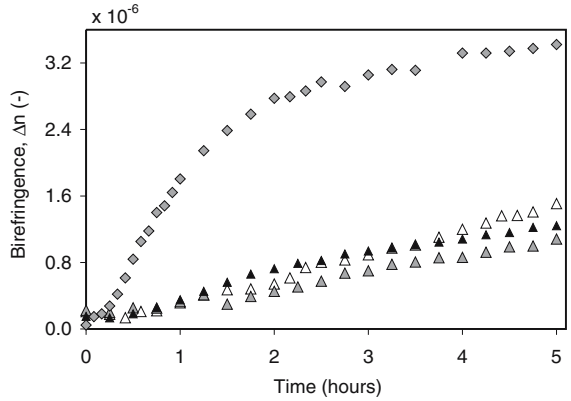

Fig. 3. Increase of birefringence during continuous shear treatment after various heating times (samples E and F; Table 1): (▲, ▲, △) 2 h, (♦) 10 h.

After 5 h of shear treatment, the birefringence signals of the sheared samples were significantly higher than the birefringence signals of the samples not subjected to shear flow, described above (Figure 2). At the start of the shear treatment (t = 0 h), no birefringence was observed for both samples (E and F), but after the start of the shear treatment, the birefringence of the samples started to increase. The increase of birefringence was faster after heating for 10 h than after heating for 2 h. The birefringence increased approximately linearly with time during the 5-h shear treatment for the sample heated for 2 h. The birefringence of the sample heated for 10 h increased very quickly during the first hour of shear treatment, but was approaching a plateau value after 5 h.

A length distribution was fitted to the birefringence decays after 5 h of shear treatment. Figure 4 shows the length distributions of the samples of 2 and 10 h heating. The three samples of 2 h heating showed a similar length distribution between which only minor variations were visible. The peak of the length distribution of the fibrils was smaller after 10 h heating than after 2 h heating.

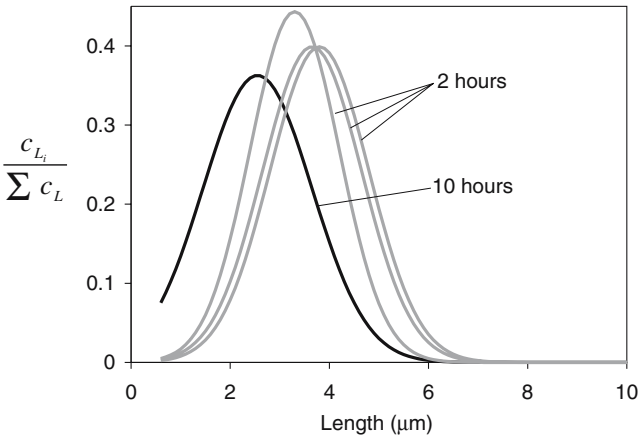

Fig. 4. Length distributions after 5 h of continuous shear treatment after 2 h heating, sample E (gray), and after 10 h heating, sample F (black).

Effect of pulsed shear treatment

In the previous section, we found that at the start of the shear treatment, no significant birefringence was observed. After the start of the shear treatment, the birefringence increased immediately; the shear flow seemed to initiate the fibril formation. To elucidate the mechanism of this effect, we varied the shear treatment. Instead of continuous shear flow, samples were subjected to transient flows, using "pulses" of shear flow at variable intervals. Each pulse consisted of 30-s flow at a shear rate of 200 s^{-1}. Three variations of pulsed shear treatment were performed as shown in Table 1 (samples G, H, and I).

Figure 5 shows the results of the pulsed shear treatments together with the average value of three experiments of continuous shear treatment (Figure 3). Sample G was given pulses each hour starting at t = 0 h. Sample H received one pulse at t = 0 h. During the 30-s shear pulses, the birefringence did not increase significantly, meaning that the fibril length concentration did not increase during the pulse treatment. After 5 h of pulsed shear treatment, the birefringence signals of samples G and H were comparable to the average birefringence signal after continuous shear treatment (sample E). This shows that the total length concentrations of the three different treatments were similar. Sample G showed a linear increase of birefringence with time over 5 h, just like the continuously sheared samples. From the decay of the birefringence after cessation of the flow, the length distributions of

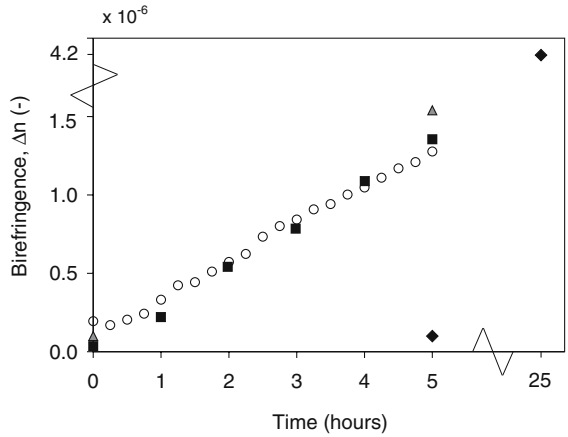

Fig. 5. Increase of birefringence of four different shear treatments: (■) shear pulses every hour (sample G); (▲) a pulse at t = 0 h (sample H); (♦) a pulse after 5 h (sample I); (○) continuous shear treatment (average of three experiments).

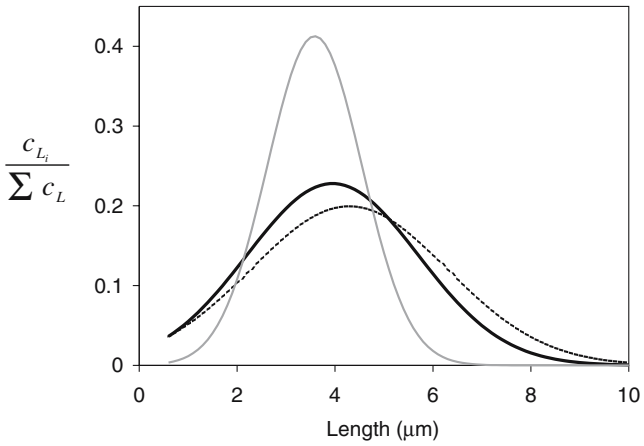

Fig. 6. Length distributions after 5 h of pulsed and continuous shear treatment (samples G, H, and D; Table 1): shear pulses every hour (solid black); a pulse at t = 0 h (dotted black); continuous shear (solid gray) (average of three experiments).

samples G and H were fitted (Figure 6). The length distributions of the two pulsed shear treatments did not differ significantly. The variances of the length distributions of pulsed shear treatments were larger than the variance of continuous shear treatment.

Another sample (sample I; Table 1) was given its first shear pulse after a lag time of 5 h. The birefringence of this sample was measured after 25 h (Figure 5). After 5 h, this sample showed negligible birefringence; few fibrils were present in the sample. However, after 25 h, the birefringence had increased to a value much higher than the sample of a comparable lag time (22.5 h) with no shear (Figures 2 and 5).

Discussion

In the previous section, it was shown that shear flow significantly enhances the formation of β-lactoglobulin fibrils. Samples that were subjected to continuous shear flow showed a significant increase of birefringence almost immediately after the shear treatment started (Figure 3). This is in contrast with a sample that was kept at rest, for which it took 22.5 h before a birefringence signal was measurable (Figure 2). However, we also showed that there is no difference in fibril concentration between applying continuous shear flow, giving short shear pulses every hour, or giving only one short shear pulse (Figure 5). This shows that the onset of the shear flow is the key parameter for enhancing the fibril growth. The duration of subsequent shear treatment was found to be of no influence on the enhancement of the fibril formation.

Static light scattering showed that during the pretreatment (comprising heating and cooling), aggregates were formed with a size in the range of nanometers. This size is smaller than the size of the fibrils, which are in the range of micrometers, and therefore, we will further refer to these small aggregates as preaggregates. Part of the preaggregates disintegrated during cooling, but the scattered intensity remained higher than the signal of native β-lactoglobulin molecules (Figure 1). A reversible aggregation was previously shown for β-lactoglobulin by Arnaudov et al.[13] The enhancing effect of the onset of shear flow could be caused by breakup of the preaggregates, but during cooling, the preaggregates also disintegrated, and this did not result in enhanced fibril formation. As we will argue below, the onset of shear flow seems to induce nucleation of the fibril formation.

Previous studies found that other mechanical disturbances, such as sonication[18] and agitation,[19] enhanced the fibril formation of different proteins, but during these studies, the difference between pulsed and continuous treatment was not studied. Although sonication is a common method to break up macromolecules, it can also be used to induce nucleation (i.e., an often used method in the sugar industry to control the size of the sugar crystals or to nucleate protein crystallization[25]). A mechanical disturbance, such as agitation, is also a known method to enhance the nucleation of a crystallization process.[26–28] Before crystallization occurs, the system has to enter the metastable part of the phase diagram, and a nucleation event is necessary to start the crystallization process. There are many examples where nucleation is triggered by penetrating the metastable part of the phase diagram by cooling. Alternatively, a nucleation event can also be induced by adding mechanical energy to the (metastable) system by, for example, sonication or agitation. Following Oosawa and Asakura,[29] we propose that the fibril formation is a one-dimensional crystallization-like process, where the total number of proteins converted into fibrils is a function of concentration, and not a sharp transition as is the case in classical crystallization. This view is supported by experimental evidence that was found by measuring the total conversion as a function of β-lactoglobulin concentration (results of Rogers et al., unpublished). The theory of Oosawa and Asakura predicts a smooth transition, especially in the case of fibrils with a thickness of one or two proteins, as is the case for β-lactoglobulin fibrils. In this view, we expect that the formation of preaggregates, which initiate the fibril growth, is similar to the formation of nuclei in a classical (three-dimensional) crystallization process. As a consequence, the fibrillar growth can also be enhanced by a mechanical disturbance, such as the

onset of shear flow. However, after one shear pulse had caused nucleation, we did not observe additional nucleation by giving more shear pulses at an interval of 1 h. Probably, there were not enough proteins left to be nucleated into preaggregates after the first nucleation had taken place.

We found that the shear flow itself, apart from the onset of shear flow, has no additional influence on the amount of fibrils relative to pulsed shear. This is direct proof that orthokinetic coagulation, flow enhanced aggregation, does not play a role under the conditions as in our experiments. In order for orthokinetic coagulation to become important within the fibril formation, fibrils have to exceed a certain length depending on the shear rate. It can be estimated that at a shear rate of $\sim 100\ s^{-1}$, we need to have fibrils present with a length in the order of 1 μm. In the initial stages of the fibril growth, this is certainly not the case, and the continuous shear flow itself will not enhance fibrillar growth. This situation can change when we use different experimental conditions, e.g., samples with a higher protein concentration or samples that are analyzed during later stages of the fibril formation process. We did observe differences in the length distribution after continuous or pulsed shear treatment. Continuous shear treatment resulted in a smaller variance of the Gaussian length distribution than pulsed shear treatment (Figure 6). This difference can be attributed to the homogenizing effect of continuous shear treatment.

Before applying shear flow, the solutions of β-lactoglobulin were heated. Without heating, no fibrils were formed within 5 h of continuous shear treatment. A longer heating time resulted in a faster fibril formation (Figure 3) and a slightly smaller average fibril length (Figure 4). A possible explanation is the presence of more preaggregates after a longer heating, which resulted in more competition between the preaggregates, which gave smaller but more fibrils.

Conclusions

We have shown that the onset of shear flow of sufficient magnitude is enough to initiate the formation of β-lactoglobulin fibrils. After initiation, extended shear treatment does not have any further effect on the fibril growth. Without the onset of shear flow, the initiation of the fibril formation is much slower. We propose that this process can be envisioned as a one-dimensional semicrystallization process as introduced by Oosawa and Asakura.[29] The formation of preaggregates is similar to that of the formation of nuclei and, as such, can be triggered by a mechanical disturbance, such as the onset of shear flow. We found no proof for the presence of orthokinetic coagulation in our experiments. However, this is expected to change when we change our experimental conditions toward higher protein concentrations, higher temperatures, or prolonged fibrillization times.

Acknowledgments We thank H. Gruppen and J. Vereijken for the useful discussions about this work, and we also thank H. Schaink for his advice for the static light scattering experiments. We acknowledge the Dutch research school, VLAG, for their financial support of this research. We acknowledge funding from the BBSRC for SSR, and the European Commission for an IHP grant awarded to the Food Physics Group of Wageningen University for a Marie Curie Training Site Fellowship (contract HPMT-2000-00188).

References

1. J.W. Kelly, Curr Opin Struct Biol **8**, 101 (1998)
2. J.A. Hardy and G.A. Higgins, Science **256**, 184 (1992)
3. M. Bucciantini et al., Nature **416**, 507 (2002)
4. M. Langton and A.M. Hermansson, Food Hydrocoll **5**, 523 (1992)
5. W.S. Gosal, A.H. Clark, and S.B. Ross-Murphy, Biomacromolecules **5**, 2420 (2004)
6. C. Veerman et al., Biomacromolecules **3**, 869 (2002)
7. C. Veerman et al., Int J Biol Macromol **31**, 139 (2003)
8. C. Veerman et al., Int J Biol Macromol **33**, 121 (2003)
9. D. Durand, J.C. Gimel, and T. Nicolai, Physica A **304**, 253 (2002)
10. L.N. Arnaudov and R. de Vries, Biophys J **88**, 515 (2005)
11. S.S. Rogers et al., Macromolecules **38**, 2948 (2005)
12. W.S. Gosal et al., Langmuir **18**, 7174 (2002)
13. L.N. Arnaudov et al., Biomacromolecules **4**, 1614 (2003)
14. G.M. Kavanagh, A.H. Clark, and S.B. Ross-Murphy, Int J Biol Macromol **28**, 41 (2000)
15. T. Lefevre and M. Subirade, Biopolymers **54**, 578 (2000)
16. D. Hamada and C.M. Dobson, Protein Sci **11**, 2417 (2002)
17. L. Nielsen et al., Biochemistry **40**, 6036 (2001)
18. P.B. Stathopulos et al., Protein Sci **13**, 3017 (2004)
19. W.E. Klunk, R.F. Jacob, and R.P. Mason, Anal Biochem **266**, 66 (1999)
20. W.E. Klunk, R.F. Jacob, and R.P. Mason, In: *Methods in Enzymology: Amyloids, Prions and other Protein Aggregates*, edited by R. Wetzel (Academic Press, 1999)
21. M. Doi and S.F. Edwards, J Chem Soc Faraday Trans 2 **74**, 918 (1978)
22. G. Marrucci and N. Grizzuti, J Polym Sci, Polym Lett Ed **21**, 83 (1983).
23. G. Marrucci and N. Grizzuti, J Non-Newton Fluid Mech **14**, 13 (1984)
24. L.N. Arnaudov and R. De Vries, In: *Kinetics of Fibrillar Aggregation of Food Proteins*, Chapter 4 (Ph.D. thesis of Wageningen University) (2005)
25. C.N. Nanev and A. Penkova, J Cryst Growth **232**, 285 (2001).
26. S.W. Young, J Am Chem Soc **33**, 148 (1911)
27. J.W. Mullin and K.D. Raven, Nature **195**, 35 (1962)
28. J.W. Mullin and K.D. Raven, Nature **190**, 251 (1961)
29. F. Oosawa and S. Asakura, *Thermodynamics of the Polymerization of Protein* (Academic Press, London, 1975)