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Fibril Assemblies in Aqueous Whey Protein Mixtures

SUZANNE G. BOLDER,[§,†] HANNEKE HENDRICKX,[†] LEONARD M. C. SAGIS,[†] AND ERIK VAN DER LINDEN*[,†]

DMV International b.v., P.O. Box 13, 5460 BA Veghel, The Netherlands, and Food Physics Group, Department of Agrotechnology and Food Sciences, Wageningen University, P.O. Box 8129, 6700 EV Wageningen, The Netherlands

Fibril formation in mixtures of whey proteins upon heating at pH 2 was investigated. Fibrils were found to coexist with other structures, such as spherulites. These spherulites consist of radially oriented fibrils. At total protein concentrations above 6 wt %, transparent gels were formed. Changing the ratio between the various whey proteins did not affect this gelation concentration as long as β-lactoglobulin (β-lg) was present, suggesting that β-lg was dominant in the gelation. Pure α-lactalbumin and pure bovine serum albumin did not form fibrils, nor did they gel upon heating at pH 2 and 80 °C for up to 10 h. They did however induce a decrease in the β-lg concentration needed for gel formation upon heating at pH 2. Our results suggest that β-lg is the only fibril forming protein at the conditions used and that no mixed fibrils are formed.

KEYWORDS: Heat-induced aggregation; heat-set gelation; fibrils; whey proteins; β-lactoglobulin

INTRODUCTION

Whey proteins are a mixture of proteins, often used as food ingredients because of their ability to form gels. Commercial whey protein ingredients, such as whey protein isolates (WPIs), are byproducts from, among others, cheese manufacturing. The most abundant whey protein is β-lactoglobulin (β-lg). It is a globular protein with a molecular mass of 18 400 g mol^{-1}, and a radius of about 2 nm (*1*). The isoelectric point (pI) of the protein is pH 5.1 (*2*). Besides β-lg, the WPIs contain other globular whey proteins, such as α-lactalbumin (α-lac) and bovine serum albumin (BSA). In the food industry, there is a growing demand for the use of whey proteins as functional material in food products. Commercial whey protein ingredients contain many impurities and have variable composition and are therefore generally too crude to study in a reproducible manner. For fundamental studies, whey proteins are usually isolated and investigated individually to understand their physicochemical properties and their abilities to form gels or stabilize foams and emulsions. For the food industry, on the other hand, isolated whey proteins are not as attractive due to relatively high costs of purification and implementation. To use the aggregation and gelation of whey proteins in food applications, it is necessary to study mixtures of whey proteins as present in a typical commercial WPI preparation.

The properties of an aggregate gel are dependent on the properties and the structure of the aggregates and their mutual interactions. Depending on the interactions between protein monomers, a wide variety of structures can be formed during aggregation. Examples are fibrils, flexible strands, branched structures, and "random" aggregates. Fibrils are formed upon heating proteins at pH values far from the isoelectric point and at low ionic strength, where they are highly charged. Fibrils are interesting because of their functional properties, such as gelation at very low protein concentrations (*3*, *4*). In systems where fibrillar protein aggregates are formed, large spherulites can also be observed. This is, for example, reported in the literature on amyloid fibrils in general and on insulin and β-lg specifically (*5*−*11*). Krebs et al. (*7*) studied the formation of spherulites in bovine insulin solutions at pH 2. Incubation of 10 mM solutions at 37 °C or 65 °C for 24 h resulted in the formation of spherulites that were found to contain a large quantity of radially arranged amyloid fibrils. Even for pure protein systems, coexistence of various structures is observed (*6*, *8*, *9*). The coexistence of structures becomes even more prominent in the phase behavior of protein mixtures. For whey protein mixtures, phase behavior has not been studied elsewhere. We will show here that in whey protein mixtures, heated at pH 2, fibrils, spherulites, and nonaggregated proteins coexist. Several papers have reported studies on the effect of mixing whey proteins on their aggregation and gelation behavior, especially at neutral pH (*12*−*23*). Kavanagh et al. (*24*) studied the heat-induced gelation of mixtures of α-lac and β-lg at pH 7 and 3, varying the α-lac to β-lg ratios while keeping the total protein concentration constant at 15%. They suggest that mixed or coupled gel networks are formed by heating the whey protein mixtures. No answer is given as to whether the aggregates are composed of one protein and form a coupled network by cross-linking the protein aggregates or whether nonspecific aggregation occurs, forming mixed aggregates that form a network.

* Corresponding author. Tel: +31 317 485515. Fax: +31 317 483669. E-mail address: erik.vanderlinden@wur.nl.
[§] DMV International b.v.
[†] Wageningen University.

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In this study, we focus on aqueous whey protein systems at pH 2, where the proteins form fibrils. Not much is known about the aggregation and gelation behavior of whey proteins other than β-lg at pH 2. Ikeda and Morris (*25*) studied the heat-induced gelation of both β-lg and WPI at pH 2 at 80 °C. The samples were studied using atomic force microscopy. Fine stranded aggregates were formed at pH 2. The diameter of the strands formed by β-lg was about 4 nm and that for WPI about 10 nm. This difference might indicate that the WPI strands were composed not only of β-lg or that the presence of other proteins might have changed the mechanism of aggregation of β-lg (*25*). Veerman et al. (*26*) studied the gelation of BSA at pH 2 for various ionic strengths and heating times. They reported that at pH 2 an ionic strength of at least 0.25 M was needed to form a gel at 6 wt % BSA. For BSA at pH 2 and various ionic strengths, polydisperse semi-flexible fibrils with a contour length of 100−300 nm were observed using transmission electron microscopy (*26*). Goers et al. (*27*) showed that α-lac forms fibrils when incubated at pH 2 in the presence of 0.025 or 0.15 M NaCl at 37 °C with constant stirring. At pH 2, α-lac adopts an acid-induced molten globule state, which has been shown to be susceptible to fibril formation (*27*). This fibril formation was accompanied by a dramatic increase in β-structure, as was determined with Fourier transform infrared spectroscopy. At increased incubation temperature (to 55 °C), the intact α-lac still formed fibrils (*27*).

In this paper, we study the effect of mixing various globular whey proteins on the structures formed upon heating samples at pH 2 and low ionic strength. We determine the phase behavior of these mixtures. Partial state diagrams are determined for aqueous mixtures of β-lg and α-lac and for aqueous mixtures of β-lg and BSA. We use the term "state diagram" here instead of "phase diagram", since not all of the phases we identify are necessarily at thermodynamic equilibrium. We will show that the phase behavior of the whey protein mixtures differs from the phase behavior of pure β-lg.

EXPERIMENTAL PROCEDURES

Sample Preparation. β-Lactoglobulin (β-lg) (product no. L0130) and bovine serum albumin (BSA) (product no. A4503) were obtained from Sigma. BioPURE - Alphalactalbumin (α-lac) and BiPRO whey protein isolate (WPI) were obtained from Davisco Foods International, Inc. (Le Sueur, MN). The BioPURE - Alphalactalbumin sample consisted of 78% α-lac, 10% β-lg, 7% BSA, and 5% immunoglobulins or glyco-macro peptides, as determined with HPLC (see section below for details). Pure α-lactalbumin (obtained from Sigma, L-6010, lot. no. 78H7024) was also used, but only for experiments with pure samples and not mixed with other whey proteins. All the other chemicals used were of analytical grade.

The protein powders were dissolved in a HCl solution of pH 2. The protein solutions were subsequently adjusted to pH 2, using a 6 M HCl solution. The protein solutions were then centrifuged at 14 000 rpm (22 600 × *g*) for 30 min using a Sorvall RC-5B refrigerated superspeed centrifuge. To remove any traces of undissolved protein, the supernatant was filtered through a protein filter (FD 30/0.45 μm Ca−S from Schleicher & Schuell). The β-lg, α-lac, and BSA samples were repeatedly diluted with HCl solution of pH 2 and filtered through a 3 K filter in an Omegacell membrane stirred cell (Filtron Technology Corp.) at 4 °C. The purpose of this filtration step was to remove traces of calcium ions and to obtain a solution with the same ionic strength and pH as the solvent. The WPI was dissolved at pH 2, and centrifugation and filtration were used to remove undissolved protein. This sample will be referred to as WPI or nonpurified WPI. We also investigated WPI samples that were subject to an additional purification step in which the whey protein aggregates were removed at a pH close to the isoelectric point (purified WPI). The removal of whey protein aggregates was done as described by Weinbreck et al. (*28*). The WPI powder was dissolved and brought to pH 4.75, using a 6 M HCl solution. The WPI solution was centrifuged for 30 min at 22 600 × *g* and subsequently filtered through 0.45 μm protein filters. The protein concentrations for β-lg, α-lac, BSA, WPI, and purified WPI were determined using a UV spectrophotometer (Cary 50 Bio, Varian) and calibration curves of known protein concentrations at a wavelength of 278 nm.

Table 1. Composition of the Whey Protein Samples in Percent of the Total Protein Concentration, as Determined with HPLC

sample	β-lg	α-lac	BSA	IgG/GMP
β-lg	100	0	0	0
β-lg/α-lac = 90/10	81.6	8.8	6.7	2.9
β-lg/α-lac = 80/20	71.9	18	7.1	3
β-lg/BSA = 90/10	90	0	10	0
β-lg/BSA = 83/17	83	0	17	0
β-lg/BSA = 70/30	70	0	30	0
WPI	51	22.7	24.8	1.4
purified WPI	63	27	8.1	1.8

High-Performance Liquid Chromatography (HPLC). The composition of the whey protein samples was analyzed by HPLC, using a Shodex KW 803 column at a flow rate of 0.6 mL/min (eluent 60% MilliQ water, 40% acetonitrile, and 0.1% trifluoroacetic acid) with detection at a wavelength of 220 nm. The samples were diluted with eluent containing 8 M urea and filtered over 0.22 μm membranes. For data collection and calculation of the separate whey proteins in the samples, PC1000 software (Thermo Separation Products GmbH, Darmstadt, Germany) was used. The amounts of the separate whey proteins were calculated using the area under the graphs, as calculated with the software, after calibration with external standards.

Phase Behavior Studies. Samples with varying total protein concentrations were prepared by diluting the concentrated stock solutions of the proteins with HCl solution of pH 2. For all proteins, the phase behavior was studied as a function of the total protein concentration. Also, mixtures of both β-lg with α-lac and β-lg with BSA were prepared by mixing the diluted stock solutions in the required ratios. Sealed glass test tubes containing the samples were heated in a water bath at 80 °C for 10 h. After the test tubes were cooled in ice−water, the samples were visually inspected for presence of structure, viscosity, turbidity, and homogeneity and analyzed with a light microscope with polarized light (Nikon 600E Eclipse, with a JVC 3-CCD video camera model KY-F55BE). Also an additional first-order red compensator was used with a polarization of 90° to examine the orientation of the fibrils in the spherulites. The cooling step had no visible effect on the phase states that were observed in the samples.

Transmission Electron Microscopy (TEM). For TEM, protein samples were prepared by heating them in sealed test tubes at pH 2 for 10 h at 80 °C. After cooling, the samples were diluted to 0.05 wt % protein in HCl solution of pH 2. The TEM samples were prepared by negative staining. A drop of the diluted sample was deposited onto a 5 nm thick carbon support film on a copper grid (400 mesh). The excess was removed after 15 s, using a piece of filter paper. A droplet of staining solution (2% potassium phosphotungstate at pH 5.6 or 2% uranyl acetate at pH 3.8) was added, and again the excess was removed after 15 s with filter paper. Electron micrographs were made using a Philips CM 12 transmission electron microscope, operating at 80 kV.

RESULTS AND DISCUSSION

Sample Composition. The composition of the samples was obtained through HPLC analysis. The compositions are given in Table 1. From Table 1, it is clear that the β-lg/α-lac samples also contained BSA and immunoglobulins or glyco-macro peptides (IgG/GMP). This was due to the composition of the α-lac sample that we used (see Experimental Procedures section). The WPI sample had a different whey protein composition after purification than before purification. In the purification step, mainly BSA was removed. The BSA that was

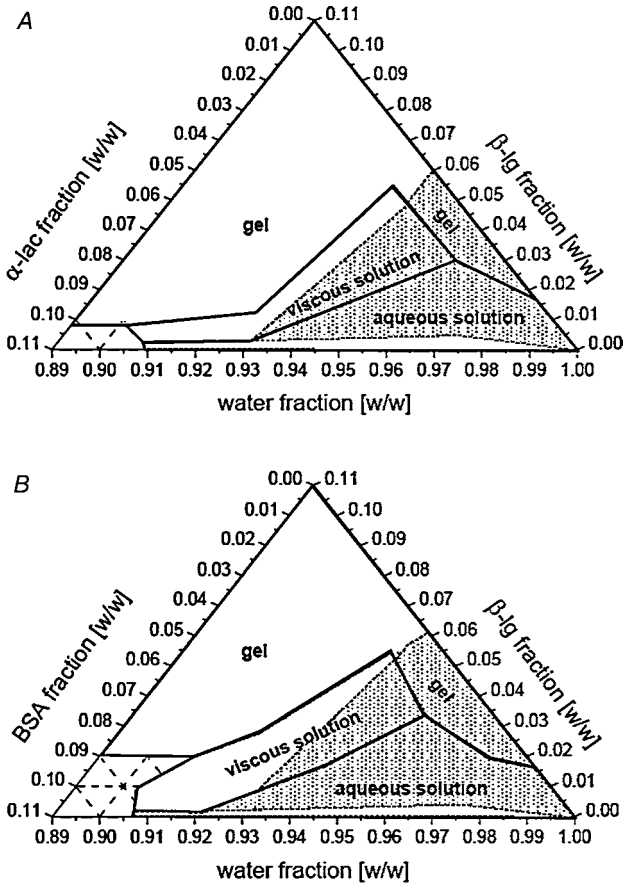

Figure 1. State diagrams for (**A**) mixtures of β-lg and α-lac and (**B**) mixtures of β-lg and BSA, both for heating the solutions for 10 h at 80 °C at pH 2 and subsequently cooling them prior to examination. The shaded areas indicate systems with macroscopically visible spherulites embedded in a continuous phase. The actual data points these diagrams are based on are available as Supporting Information.

removed was most likely present in aggregates, which were precipitated at pH 4.75 and removed during centrifugation.

State Diagrams and Structures of Protein Assemblies. *State Diagrams.* The phase behavior for mixtures of whey proteins as a function of the ratio between the proteins and the total protein concentration was determined in the aqueous-rich corner of the state diagrams. **Figure 1** shows the state diagrams for mixtures of β-lg/α-lac mixtures (**A**) and β-lg/BSA mixtures (**B**). The spherical structures will be referred to as spherulites, analogous to the similar ordered structures found in polymer crystallization. These structures were reported previously in the literature on β-lg at low pH (*5*, *8*) and on insulin at low pH. (*7*, *10*, *11*, *29*, *30*) For samples with a high β-lg to α-lac or β-lg to BSA ratio, gels with embedded spherulites were formed at total protein concentrations between 2 and 6 wt %. For samples with a similar total protein concentration but with a lower β-lg to α-lac or β-lg to BSA ratio, aqueous solutions or viscous solutions with spherulites were obtained for total protein concentrations up to 6 wt %. By aqueous solutions, we mean solutions with a low viscosity, approximately equal to that of water. The distinction between viscous and aqueous solutions was made by visual observation only. Clear gels, free of spherulites, were found at total protein concentrations above 6 wt %, independent of the β-lg to α-lac or β-lg to BSA ratio, as long as sufficient β-lg (about 70% of the total protein content) was present in the samples. At very low β-lg/α-lac or β-lg/BSA ratios, the gelation concentration was found to increase

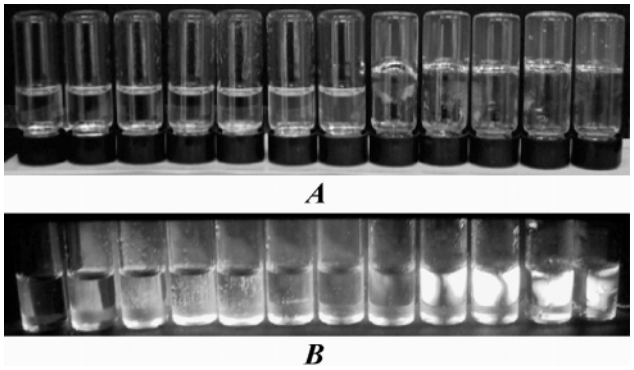

Figure 2. Glass test tubes with purified WPI samples after heating for 10 h at pH 2 and 80 °C and subsequent cooling to room temperature. From left to right the samples shown are 0.5, 1, 1.5, 2, 3, 4, 5, 6, 7, 8, 9, and 10 wt %. In panel **A**, test tubes with the samples were turned upside down to indicate which samples had gelled. In panel **B**, test tubes with the samples were placed up-right between crossed polarizers to study their birefringence.

to higher total protein concentrations (>6 wt %). Neither pure α-lac nor pure BSA formed a gel under the given conditions for concentrations up to 9 wt %. No fibrils or spherulites were formed upon heating pure α-lac or pure BSA, as was observed with light microscopy and TEM. This is described in more detail in the next section. Addition of α-lac or BSA to β-lg induced a decrease in the β-lg gelation concentration. Based on these results, we conclude that β-lg is responsible for the heat-set gelation of whey protein samples at pH 2. The phase behavior observed for WPI (either purified or nonpurified WPI), as a function of the total protein concentration, did agree with the behavior observed for the mixtures as shown in **Figure 1**.

For constructing the state diagrams, samples were studied with polarized light. In **Figure 2**, an example is given for the macroscopically observed samples, in this case for heated purified WPI. The same observations were made for the other whey protein mixtures. In **Figure 2A** test tubes with the heated samples are shown, turned upside down, showing that from 6 wt % and up semi-transparent gels were formed. The increase in turbidity is due to the increasing protein concentration in the samples. The turbidity was not affected by the heat treatment. In **Figure 2B**, these same test tubes were placed up-right between crossed polarizers, showing birefringence from spherulites in the samples between 1 and 6 wt % (the spherulites light up brightly when placed between crossed polarizers). When these samples are shaken flow birefringence is observed throughout the entire sample. The strong gels formed in the samples of 7 wt % and up showed permanent birefringence throughout the entire sample. We have also observed that these gels are strain-thinning (results not shown). These observations (permanent birefringence and strain-thinning behavior) suggest that these samples, which we refer to here as gels, are possibly liquid crystalline phases. The transition we have so far referred to as gelation appears to be a transition from a concentrated fibril solution (showing only flow birefringence) to a polydomain liquid crystalline phase with permanent birefringence. This was previously suggested by Sagis et al. (*8*) for β-lg samples that were heated at pH 2 for 10 h at 80 °C at concentrations above 9.5 wt %. The theory of Odijk (*35*) predicts such a transition for semi-flexible chains in the same range where we observe it.

Structures of Protein Assemblies. The spherulites that were observed with the naked eye showed a Maltese cross extinction pattern when viewed between crossed polarizers (see also

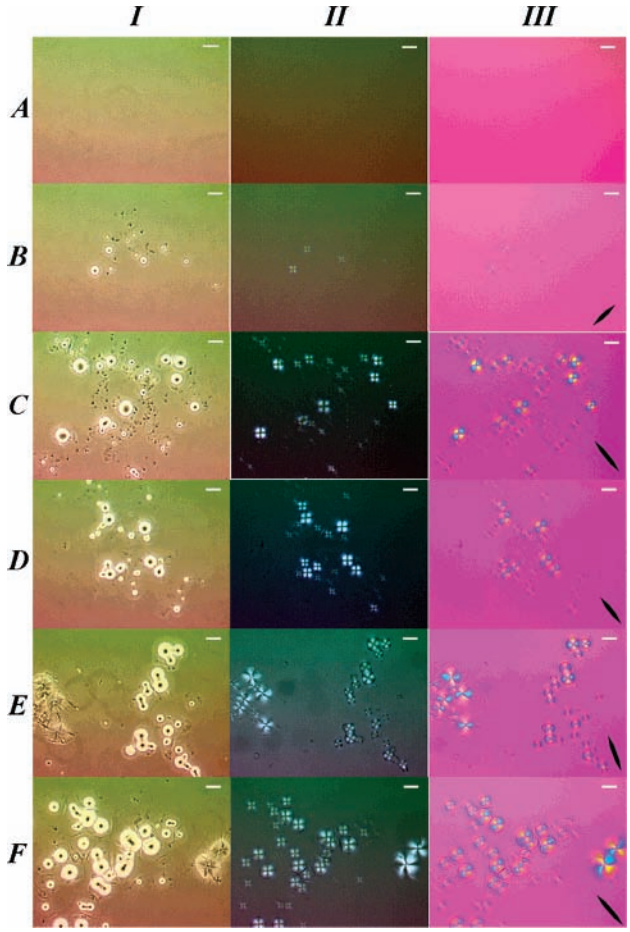

Figure 3. Microscopic images of 2 wt % purified WPI samples at pH 2 that were heated at 80 °C for 2, 4, 6, 8, 10, or 34 h, respectively, for rows **A**–**F**. Column **I** shows images for bright field, column **II** shows images with polarized light, and column **III** shows images with polarized light and a first-order red compensator filter. All scale bars represent 100 μm. The double-headed arrows indicate the orientation of the fast optical axis of the compensator filter, which was always at a 45° angle with respect to the transmission orientation of the polarizer and the analyzer.

Figures 3 and **4**). This indicates that the structures present had some kind of crystalline order. The spherulites fracture when a sufficiently high force is applied, using a high shear mixer (Ultra-Turrax), without any observable deformation (results not shown). This suggests that the spherulites are crystalline, in contrast to the bulk phases which are liquid crystalline. No differences were observed between the spherulites that were obtained in the various samples studied, regardless of the type of proteins present. To study the development of the spherulites as a function of time, test tubes were filled with 2 wt % purified WPI solution at pH 2. The test tubes were sealed and placed in a water bath at 80 °C. After varying heating times (2, 4, 6, 8, 10, or 34 h), the test tubes were taken out of the water bath and cooled. Subsequently the samples were examined both visually and with a light microscope, using bright field, polarized light, or polarized light combined with a first-order red compensator filter. In **Figure 3**, microscopic images of the samples are shown, which clearly show that spherulites are formed upon heating. After 2 h of heating, some vague sphere-like structures were visible with bright field (**Figure 3A**, **column I**), but no structure was observed with polarized light. Upon longer heating times, the number and size of spherulites increased, reaching a size between about 20 and 100 μm. They show a Maltese cross extinction pattern when viewed between crossed polarizers, as

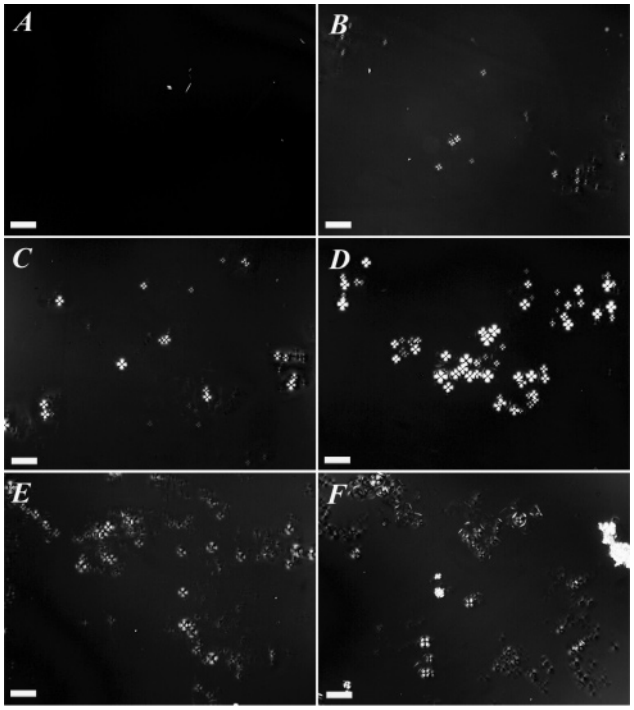

Figure 4. Microscopic images of (**A**) 0.5, (**B**) 1, (**C**) 2, (**D**) 3, (**E**) 4, and (**F**) 5 wt % WPI. All samples were heated for 10 h at pH 2 and 80 °C. Scale bars represent 200 nm.

can be seen in **Figure 3** (**columns II** and **III**). The samples that were heated for 10 or 34 h (**Figure 3E,F**) also showed some larger spherulites (>100 μm). These larger spherulites had a slightly nonspherical shape, when viewed in bright field (see **Figure 3E,F**, **column I**). In polarized light, a somewhat distorted Maltese cross is observed, which is also indicative of a nonspherical shape. The images that were taken using polarized light combined with the first-order red compensator filter (**Figure 3**, **column III**) show that quadrants of the spherulites are colored. The blue color indicates that the fast optical axis of the spherulite is parallel to the fast optical axis of the first-order red compensator filter.

When spherulite formation is compared among various WPI concentrations, an increasing number of spherulites was observed between 1 and 3 wt %, followed by a decrease for 4 and 5 wt %. This is shown in **Figure 4**. In the WPI samples with concentrations of 2 wt % and higher, isotropic structures between the spherulites are visible in bright field, as is also visible in **Figure 3**, **column I**. The structures must be isotropic since they do not show any birefringence when placed between crossed polarizers. These isotropic structures grow with increasing WPI concentration, until a gel (or liquid crystalline phase) without spherulites is formed at total protein concentrations of 6 wt % and higher. The reason for the disappearance of the spherulites may simply be a kinetic effect. Spherulite formation is very slow (see **Figure 3**), and when the transition to a liquid crystalline or gel-like phase, which is fast at high concentrations, occurs before the spherulites have formed, their formation will be inhibited.

The average diameter of the spherulites was constant for various concentrations, ranging between about 20 and 100 μm. We have observed that the spherulites are very stable, for periods of time up to over a year (results not shown). These observations are in line with recent work of Rogers et al. (*36*). They observe that the fibrils grow radially from an amorphous core, which does not decrease in size.

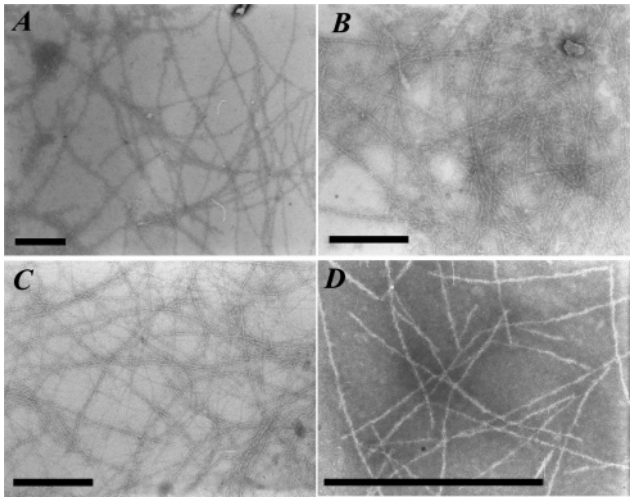

Figure 5. TEM micrographs of (**A**) 2.5 wt % β-lg, (**B**) 2.5 wt % β-lg/BSA mixture with ratio β-lg to BSA of 90/10, (**C**) 1.5 wt % purified WPI, and (**D**) 2.5 wt % β-lg/α-lac mixture with ratio β-lg to α-lac of 90/10. All samples were heated for 10 h at pH 2 and 80 °C. Scale bars represent 1 μm.

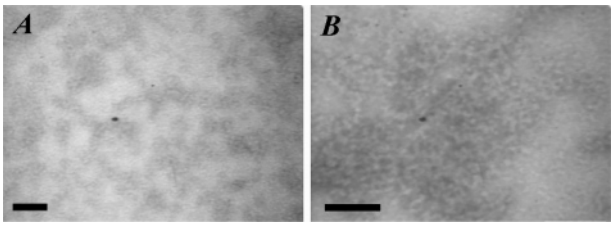

Figure 6. TEM micrographs of (**A**) pure BSA and (**B**) pure α-lac. Both samples were heated at a concentration of 2.5 wt % for 10 h at pH 2 and 80 °C. The samples were diluted to 0.05 wt % with HCl solution at pH 2. The scale bars represent 100 nm.

In all samples containing β-lg, spherulites were observed macroscopically and using a light microscope. It is known that β-lg forms fibrils under the conditions chosen for our experiments (*1*, *3*, *25*, *31*−*33*). These fibrils are about the width of a monomer and up to several micrometers long (*1*, *31*−*33*) (see also **Figure 5A**). When other whey proteins were mixed with β-lg, fibrils were still formed as observed with TEM (see **Figure 5B**−**D**). In the absence of β-lg, no fibrils or spherulites were formed by pure α-lac or pure BSA as observed visually or with light microscopy (results not shown). For BSA, this is in agreement with the results found by Veerman et al. (*26*) who reported that at pH 2 an ionic strength of at least 0.25 M was required to form BSA fibrils. Moreover, Veerman et al. (*26*) observed fibrils only after heating BSA at 60 °C and not at 80 °C (unpublished results). TEM on the α-lac and BSA samples revealed that no fibrils were formed. We observed only very small structures in the samples, as shown in **Figure 6**. These structures might be small aggregates with a diameter of 10−20 nm. Another possibility is that these structures are grains of the uranyl acetate staining solution since we observed these structures also in TEM images of other samples. Cold-gelation experiments with the addition of Ca^{2+} at pH 7 did not show any gelation, indicating that no structure was formed (*3*, *34*). In samples where β-lg is present, both fibrils and spherulites were observed. The fibrils formed in whey protein mixtures show a very similar morphology as the fibrils formed by β-lg (see **Figure 5**). Combining this with the results for pure α-lac or pure BSA where no spherulites or fibrils were formed, we suggest that the fibrils might be formed of just β-lg. This is supported by recent findings of Krebs et al. (*37*). They examined the specificity of seeding of hen lysozyme fibrils at low pH by using preformed fibrils of five different proteins. These five proteins had an increasingly different sequence identity and structure than hen lysozyme. The experiments showed that a decrease in sequence identity between hen lysozyme and the protein that was used for seeding a hen lysozyme solution decreased the efficiency of seeding (*37*). Therefore it seems very unlikely that mixed fibrils will be formed of proteins that have different sequences. Since β-lg has a very different sequence and structure than α-lac or BSA, these different proteins will most likely not be incorporated into one fibril. At higher ionic strengths, Ikeda and Morris (*25*) report results where they suggest that the WPI strands they formed are composed not only of β-lg but also other proteins or that the presence of the other proteins might modify the aggregation mechanism of β-lg. They used a much higher ionic strength, where more flexible chains are formed upon heating β-lg or WPI, compared to the semi-flexible fibrils that are formed at low ionic strength. This is also evident from the AFM images presented in their paper (*25*). It may be possible that aggregation at higher ionic strength does lead to the formation of mixed aggregates (*25*). Our results show that for low ionic strengths this is unlikely.

Effect of α-lac and BSA on Gelation. Upon heating of β-lg at pH 2, low ionic strength and 80 °C, the conversion of monomers into aggregates is lower than 100% (*1*, *31*, *33*). In heated whey protein samples, fibrils, spherulites, and non-aggregated proteins will be present and possibly small aggregates that are formed by α-lac and BSA. Depletion interactions between the long fibrils can be induced by monomers (or small aggregates). This can result in attractive interactions between β-lg fibrils, causing a decrease in the minimum gelation concentration of β-lg. This might explain the gelation in samples with high β-lg ratios and total protein concentrations as low as 2 wt %, resulting in gels with entrapped spherulites. At lower β-lg ratios but similar total whey protein concentrations (2−6 wt %), the depletion interactions will occur as well, but because of a lower fibril concentration, only viscous solutions will be formed instead of gels. At total protein concentrations of 6 wt % and higher, where no spherulites are observed, clear gels or liquid crystalline phases are formed. This is only the case when β-lg is present in the samples.

An alternative explanation for our observations could be that BSA and α-lac are affecting the conversion of protein monomers into fibrils. If for example these proteins increase the amount of protein incorporated in fibrils, a drop in the minimum gelation concentration of β-lg would be observed. Since we have not determined the actual conversion of β-lg, we cannot comment on the validity of this explanation.

Conclusion. Fibril formation and phase behavior of heated aqueous whey protein mixtures were studied for various ratios between the whey proteins and as a function of the total protein concentration. Fibrils were found to coexist with other structures, such as spherulites. Spherulites were observed for samples with total protein concentrations up to 6 wt % with β-lg present. These spherulites consist of radially oriented fibrils and are similar to the spherulites that have been observed in amyloid solutions. The amount of spherulites formed increased for increasing total protein concentrations, with a maximum at about 4 wt %. Above 4 wt %, the amount of spherulites decreased, and they completely disappeared at about 6 wt %. The spherulites grow in size and number upon increasing heating time. At total protein concentrations above 6 wt %, transparent gels were formed. Changing the ratio between the various whey

proteins did not affect this gelation concentration as long as β-lg was present, suggesting that β-lg is dominant in the heat-induced gelation of whey proteins at pH 2. Mixing of β-lg with other whey proteins, as present in commercial whey protein isolate preparations, did not show a change in the morphology of the fibrils formed upon heating at pH 2. α-Lac and BSA were both found to decrease the gelation concentration for β-lg. We propose that α-lac and BSA induce attractive interactions between the fibrils formed by β-lg. α-Lac and BSA appear to form small aggregates that can induce depletion, resulting in an attractive interaction between the β-lg fibrils. This will cause gelation at lower β-lg concentrations upon heating at pH 2 in the presence of α-lac or BSA. α-Lac and BSA alone did not form fibrils, as was determined with TEM, nor did they gel upon heating at pH 2 and 80 °C for up to 10 h. This suggests that β-lg is the only fibril forming protein at the conditions used and that no mixed fibrils are formed.

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Supporting Information Available: Detailed state diagrams for β-lg/α-lac and β-lg/BSA mixtures. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Aymard, P.; Nicolai, T.; Durand, D.; Clark, A. *Macromolecules* **1999**, *32*, 2542−2552.
(2) Verheul, M. *Aggregation and gelation of whey proteins*; Rheology Group, Universiteit Twente: Enschede, 1997; p 153.
(3) Veerman, C.; Baptist, H.; Sagis, L. M. C.; van der Linden, E. *J. Agric. Food Chem.* **2003**, *51*, 3880−3885.
(4) Veerman, C.; Sagis, L. M. C.; van der Linden, E. *Macromol. Biosci.* **2003**, *3*, 243−247.
(5) Bromley, E. H. C.; Krebs, M. R. H.; Donald, A. M. *Faraday Discuss.* **2004**, *128*, 13−27.
(6) Hamodrakas, S. J.; Hoenger, A.; Iconomidou, V. A. *J. Struct. Biol.* **2004**, *145*, 226−235.
(7) Krebs, M. R. H.; MacPhee, C. E.; Miller, A. F.; Dunlop, I. E.; Dobson, C. M.; Donald, A. M. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 14420−14424.
(8) Sagis, L. M. C.; Veerman, C.; Ganzevles, R.; Ramaekers, M.; Bolder, S. G.; van der Linden, E. *Food Hydrocolloids* **2002**, *16*, 207−213.
(9) Sagis, L. M. C.; Veerman, C.; van der Linden, E. *Langmuir* **2004**, *20*, 924−927.
(10) Waugh, D. F. *J. Am. Chem. Soc.* **1946**, *68*, 247−250.
(11) Waugh, D. F.; Wilhelmson, D. F.; Commerford, S. L.; Sackler, M. L. *J. Am. Chem. Soc.* **1953**, *75*, 2592−2600.
(12) Calvo, M. M.; Leaver, J.; Banks, J. M. *Int. Dairy J.* **1993**, *3*, 719−727.
(13) Dalgleish, D. G.; Senaratne, V.; Francois, S. *J. Agric. Food Chem.* **1997**, *45*, 5.
(14) Elfagm, A. A.; Wheelock, J. V. *J. Dairy Sci.* **1978**, *61*, 28−32.
(15) Gezimati, J.; Creamer, L. K.; Singh, H. *J. Agric. Food Chem.* **1997**, *45*, 1130−1136.
(16) Gezimati, J.; Singh, H.; Creamer, L. K. *J. Agric. Food Chem.* **1996**, *44*, 804−810.
(17) Gezimati, J.; Singh, H.; Creamer, L. K. *ACS Symp. Ser.* **1996**, *650*, 113−123.
(18) Havea, P.; Singh, H.; Creamer, L. K. *J. Dairy Res.* **2001**, *68*, 483−497.
(19) Hines, M. E.; Foegeding, E. A. *J. Agric. Food Chem.* **1993**, *41*, 341−346.
(20) Hong, Y.-H.; Creamer, L. K. *Int. Dairy J.* **2002**, *12*, 345−359.
(21) Matsudomi, N.; Oshita, T.; Kobayashi, K.; Kinsella, J. E. *J. Agric. Food Chem.* **1993**, *41*, 1053−1057.
(22) Matsudomi, N.; Oshita, T.; Sasaki, E.; Kobayashi, K. *Biosci., Biotechnol., Biochem.* **1992**, *56*, 1697−1700.
(23) Schokker, E. P.; Singh, H.; Creamer, L. K. *Int. Dairy J.* **2000**, *10*, 843−853.
(24) Kavanagh, G. M.; Clark, A. H.; Gosal, W. S.; Ross-Murphy, S. B. *Macromolecules* **2000**, *33*, 7029−7037.
(25) Ikeda, S.; Morris, V. J. *Biomacromolecules* **2002**, *3*, 382−389.
(26) Veerman, C.; Sagis, L. M. C.; Heck, J.; van der Linden, E. *Int. J. Biol. Macromol.* **2003**, *31*, 139−146.
(27) Goers, J.; Permyakov, S. E.; Permyakov, E. A.; Uversky, V. N.; Fink, A. L. *Biochemistry* **2002**, *41*, 12546−12551.
(28) Weinbreck, F.; de Vries, R.; Schrooyen, P.; de Kruif, C. G. *Biomacromolecules* **2003**, *4*, 293−303.
(29) Krebs, M. R. H.; Bromley, E. H. C.; Donald, A. M. *J. Struct. Biol.* **2005**, *149*, 30−37.
(30) Waugh, D. F. *J. Am. Chem. Soc.* **1948**, *70*, 1850−1857.
(31) Arnaudov, L. N.; de Vries, R.; Ippel, H.; van Mierlo, C. P. M. *Biomacromolecules* **2003**, *4*, 1614−1622.
(32) Gosal, W. S.; Clark, A. H.; Pudney, P. D. A.; Ross-Murphy, S. B. *Langmuir* **2002**, *18*, 7174−7181.
(33) Veerman, C.; Ruis, H.; Sagis, L. M. C.; van der Linden, E. *Biomacromolecules* **2002**, *3*, 869−873.
(34) Bolder, S. G.; Hendrickx, H.; Sagis, L. M. C.; van der Linden, E. *Appl. Rheol.*, submitted for publication.
(35) Odijk, T. *Macromolecules* **1986**, *19*, 2313−2329.
(36) Rogers, S. S.; Krebs, M. R. H.; Bromley, E. H. C.; van der Linden, E.; Donald, A. M. *Biophys. J.* **2006**, *90*, 1043−1054.
(37) Krebs, M. R. H.; Morozova-Roche, L. A.; Daniel, K.; Robinson, C. V.; Dobson, C. M. *Protein Sci.* **2004**, *13*, 1933−1938.

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