Interfacial Activity and Interfacial Shear Rheology of Native β-Lactoglobulin Monomers and Their Heat-Induced Fibers

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Interfacial properties of native β-lactoglobulin monomers and their heat-induced fibers, of two different lengths, were investigated at pH 2, through surface tension measurements at water−air and water−oil interfaces and interfacial shear rheology at the water−oil interface. The applied heat treatment generates a mixed system of fibers with unconverted monomers and hydrolyzed peptides. The surface tension of this system at the water−air interface decreased more rapidly than the surface tension of native monomers, especially at short times (10^{-3} to 10^{2}s). This behavior was not observed when the unconverted monomers and peptides were removed by dialysis. At the water−oil interface, the adsorption kinetics was much faster than at the water−air interface, with a plateau interfacial pressure value reached after 1 h of adsorption. For all the systems, interfacial shear rheology showed the formation of a highly elastic interface, with solid-like behavior at $1-10^{3}$ s time scales. The highest modulus was observed for the long fibers and the lowest for the native monomers. Creep−compliance curves in the linear regime could be reduced to a single master curve, showing similar spectra of relaxation times for all investigated systems. Upon large deformations, the interfaces formed with long fibers showed the most rigid and fragile behavior. This rigidity was even more pronounced in the presence of unconverted monomers.

1. Introduction

Protein adsorption is a complex phenomenon, where adsorption, desorption, unfolding, and aggregation occur simultaneously at the interface.[1] Fundamental work devoted to the characterization and understanding of the interfacial activity of food proteins, their adsorption mechanisms and kinetics, the nature and behavior of their structure at the interface, and their importance in foaming or emulsifying performance, has been carried out extensively.[2−13] In particular, several papers highlighted that a slight modification of the protein by a chemical,[3] physical,[8,13] or enzymatic treatment[5] could enhance adsorption kinetics and improve interfacial properties. Following such treatments, the faster adsorption kinetics can be attributed to an increase of protein hydrophobicity and/or formation of hydrolyzed peptides, which can lower the kinetic barrier to adsorption experienced by native proteins at the interface.[14,15]

Heat treatment of β-lactoglobulin, the major whey protein, at different pH and different ionic strengths leads to the formation of supramolecular aggregates having various structures, ranging from spherical to rod-like structures.[16] Heat-induced particulate whey protein aggregates obtained at neutral pH have been investigated for their interfacial properties.[10,11,13,17] In particular, Zhu and Damodaran[13] showed that mildly heat-treated whey protein isolate had better foamability and foam stability than the native protein, and a higher hydrophobicity. They also investigated mixtures of aggregates with native proteins and showed that the native/aggregated protein ratio could be optimized to achieve the best foamability and foam stability.

Heat treatment of β-lactoglobulin at acidic pH and low ionic strength leads to the formation of rod-like aggregates with a high aspect ratio (1−20 μm long and 4 nm as average diameter).[16] So far, no studies have been carried out on the interfacial properties of such aggregates. However, it is of particular interest to investigate the interfacial properties of heat-induced β-lactoglobulin fibers since their structure and dimension are very different from heat-induced particulate aggregates. Indeed, several papers highlighted the outstanding stabilizing properties of rod-like particles for both water−oil and water−air interfaces.[18−21] For example,

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in foams, resin-based microrods significantly delayed drainage and enhanced foam stability, and these properties were explained by the formation of a thick protective shell around the air bubbles, by entanglement and overlapping of the rods, something which obviously cannot be achieved by spherical particles.[18]

In addition to classical foaming/emulsifying experiments, interfacial shear rheology provides a finer insight into deformation and flow properties of protein-adsorbed interfaces.[22,23] The study of such properties, upon imposition of stress, is also relevant for understanding and optimizing stabilization of foams and emulsions. From the literature, it is usually admitted that one important stabilization mechanism provided by globular proteins at the interface is the formation of a strong viscoelastic layer of low compressibility, which protects the emulsion/foam droplets against growth/shrinkage[24] and can delay drainage and resist stretching of the interface.[22] This viscoelastic behavior has often been attributed to the formation of a physical gel-like network held by covalent bonds. For instance, it was shown that adsorbed β-lactoglobulin at the water−oil interface at neutral pH polymerized through the formation of covalent disulfide bridges exclusively.[25] However, the surface shear behavior was shown to be mainly dependent on physical interactions rather than on chemical ones.[26]

Proteins at the interface interact through physical interactions such as electrostatic, hydrophobic, and van der Waals type. For instance, close to the isoelectric pH, in the absence of electrostatic repulsive interactions, proteins at the interface experience mainly attractive interactions, which would lead to aggregation and formation of a network.[27−29] Besides this mechanism, which is known to form interfacial gels, it has been proven that repulsive interactions can also result in a gel-like interface, which is rather a glassy 2D system where the proteins are sterically jammed.[24,30−33] Slowing down of protein diffusion at the interface was also observed for very low surface concentration, far below the monolayer saturation concentration.[24] The interfacial properties of protein aggregates, in particular, rod-like aggregates, are expected to be more complex to interpret than those of the native protein, since additional parameters are to be taken into account, such as the aspect ratio.

In this work, we investigate the interfacial activity and rheology of native β-lactoglobulin and heat-induced β-lactoglobulin fibers at the water−oil interface. The experiments were performed at pH 2, that is, the pH at which β-lactoglobulin fibers are formed. At pH 2, native β-lactoglobulin is present as a monomer, and has the highest net charge, bearing only positively charged or neutral residues. The SH group from the cystein residue is not active; thus, no formation of intermolecular disulfide bridges is expected. In the literature, it was also shown that, upon adsorption of β-lactoglobulin monomers at the oil−water interface at pH 2, no covalent bonds are created.[22] Heat-induced β-lactoglobulin fibers have a similar protein net charge as the native monomer.[16] Heat treatment at pH 2 yields a conversion of native β-lactoglobulin into fibers, which is lower than at higher pH and results in a mixture of fibers with unconverted monomers whose amount can be significant. With our heating protocol,[34] the conversion rate is 75%, so the 25% unconverted monomers cannot be neglected.

The initial bulk concentration chosen for our work was 0.1% wt. At this concentration, β-lactoglobulin fibers in bulk water are in an isotropic state, meaning that there is no ordering prior to adsorption at the interface. In fact, from our previous work on the phase diagram of β-lactoglobulin fibers in water, at pH 2, the isotropic−nematic phase transition was found to occur at 0.4 wt % and gelation at 5 wt %.[34] Although one can expect some ordering at the interface, this was not characterized in the present work, due to the complexity of the system. Models for predicting a 2D alignment at an interface would require monodisperse colloidal samples, with a well-defined linear charge density, a perfectly rigid behavior, and a known surface concentration.

Interfacial activity of native β-lactoglobulin and heat-induced β-lactoglobulin fibers was assessed by drop tensiometry at the water−oil interface. A complementary surface tension measurement was carried out at the water−air interface, providing insight into adsorption mechanisms at very short times (10^{-3} s) using a maximum bubble pressure tensiometer. Indeed, this experiment shed details on the very fast adsorption kinetics, which were not visible with drop tensiometry. In addition, the slower kinetics expected at the water−air interface compared to the water−oil interface[22] would allow a better understanding of the adsorption steps of the native proteins/fibers at the interface.

The rheological properties of adsorbed protein layers were investigated by interfacial shear rheology at the water−oil interface. The protein-adsorbed interface was left to age for 16.7 h before it was characterized for its rheological properties. The linear viscoelastic behavior was investigated in detail for all systems. At last, the nonlinear behavior was also characterized by applying a constant macroscopic strain rate. The investigation of the type of β-lactoglobulin fibers was performed by including in the study two different fiber types and lengths: a long, semiflexible type (contour length $\gg$ persistence length), directly generated from the heat treatment, and a short, rigid type (contour length $\leq$ persistence length), obtained by cutting the long ones using a high-pressure homogenizer. Furthermore, the effect of fiber purity was also investigated, by considering different grades of fibers ranging from processed fibers inclusive of unconverted monomers to dialyzed fibers, when unconverted monomers have been removed. Unexpectedly, surprising similarities between the systems in the linear rheological behavior were evidenced and characterized, in contrast to the nonlinear behavior.

2. Materials and Methods

2.1. Materials. *2.1.1. Preparation of Demineralized β-Lactoglobulin Powder.* BioPURE β-lactoglobulin (lot JE 003−6−922) was obtained from Davisco Foods International, Inc. (Le Sueur, MN). The powder consisted of 97% protein on a dry basis, among which 95.9% of β-lactoglobulin (variant A, 55.6%; variant B, 44.4%) as determined by HPLC (data not shown). To remove non-"native" proteins, the protein powder was dissolved in Milli-Q water at 10 wt % concentration and the

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Table 1. Experimental Conditions for Production of Long, Semiflexible β-Lactoglobulin Fibers

β-lactoglobulin concentration (% wt)	2
pH	2.0
temperature (°C)	90
volume (mL)	100
stirring	yes
heating time (min)	300
conversion rate (%)	75 ± 3

solution was adjusted to pH 4.6 using a 1 M HCl solution. Then, it was centrifuged at 15 000 rpm for 15 min at 20 °C using a Sorvall Evolution RC high speed centrifuge (Thermo Fisher Scientific, Germany), and the supernatant was recovered and adjusted to pH 2 using a 1 M HCl solution. To further remove possible residual traces of insoluble proteins, the supernatant was filtered through a 0.22 μm Millipore filter. To remove traces of ions that could affect β-lactoglobulin aggregation,[35] the filtered protein solution was dialyzed first against pH 2 Milli-Q water and then against Milli-Q water again, using a Spectra-Por Dialysis Membrane 1, with a MWCO of 6000−8000 Da (Spectrum Laboratories, Inc., CA). Dialysis tubes were previously boiled for 10 min in demineralized water in the presence of 1 mM EDTA and extensively rinsed with demineralized water. The volume ratio between the solvent and the protein solution was kept constant at ~40 during dialysis. The dialysis was performed at 4 °C with at least 4 h between the dialysis buffer changes. After dialysis, the solution was adjusted back to pH 2. The mineral composition of β-lactoglobulin was determined before and after dialysis by ICP-EAS in a previous work.[16] The protein solution was finally freeze−dried and placed in a desiccator at room temperature for storage. All the experiments were carried out using this dialyzed and freeze−dried β-lactoglobulin powder.

2.1.2. Preparation of Long β-Lactoglobulin Semiflexible Aggregates. Dialyzed and freeze−dried β-lactoglobulin powder was dissolved in Milli-Q water at room temperature, centrifuged at 10 800 g for 1 h at 20 °C using a Sorvall RC3C Plus Centrifuge (DuPont, Newtown, CT), adjusted to the proper pH, and filtered through a 0.45 μm Millipore filter before heat treatment. The experimental conditions we use in this work were adapted from a previously developed protocol[16,34,36] and are summarized in Table 1.

The conversion rate is the amount of β-lactoglobulin monomers which have been converted into aggregates after heating. The protocol for its determination was adapted from the protocol of Veerman et al.[37] and was described in a previous work.[16] We chose to work with 2 wt % solutions, as at this concentration a high conversion of β-lactoglobulin monomers into fibers can be achieved while avoiding the formation of spherulites, which typically appear in competition with fiber formation during the heating process at larger concentrations.[38−40]

The 2% β-lactoglobulin solution was distributed in 100 mL flasks, which were hermetically sealed and placed in a water bath for heating. Each flask contained a magnetic bar to stir the solution during the heating process, as stirring enhanced the conversion rate and the birefringence and reduced the formation of spherulites.[41] After heating, the flasks were immediately cooled by immersion in ice−water mixtures to quench the aggregation process. The solution was checked by eye for transparency and absence of spherulites. These long fibers have a diameter of approximately 4 nm, a persistence length of between 1 and 3 μm,[42] and a contour length of 1−20 μm.[16,42]

Table 2. Summary of the β-Lactoglobulin Systems Investigated in This Work

	long fibers	short rods	monomers	rod/monomer ratio
1. processed long fibers	X		X	75/25
2. dialyzed long fibers	X			100/0
3. processed short rods		X	X	75/25
4. dialyzed short rods		X		100/0
5. monomers			X	0/100

From now on, we refer in this manuscript to this system as "processed long fibers". This system is a mixed system of approximately 75% long fibers and 25% unconverted monomers and partially hydrolyzed peptides.

2.1.3. Preparation of Pure Long Fibers by Dialysis. In order to study a system of pure semiflexible long fibers, dialysis was performed on the solution of 2% long fibers to remove the unconverted monomers and hydrolyzed peptides. The dialysis was performed against pH 2 Milli-Q water, using a Cellulose Ester Spectra-Por dialysis membrane having a MWCO of 100 000 Da (Spectrum Laboratories, Inc., CA). The volume ratio between the solvent and the protein solution was kept constant at around 80 during the dialysis. The dialysis was performed at 4 °C, with at least 4 h between the dialysis buffer changes. The efficiency of the dialysis was estimated by measuring the concentration of the monomers in the dialysis bath via absorbance measurements at 278 nm. The concentration was determined using a calibration curve drawn for β-lactoglobulin monomers in pH 2 water from 0.001% to 0.1% wt. The extinction coefficient was 0.828 22 L. $cm^{-1}.g^{-1}$ and was in good agreement with the value determined by Arnaudov et al.[43]

The dialysis process allowed a decrease in the monomer concentration by about 500 times, i.e., the final concentration of the monomer was less than 0.001%. Note that this dialysis removes both unconverted monomers and the peptides, leaving a pure system with long, semiflexible fibers only. From now on, we refer in this manuscript to this system as "dialyzed long fibers".

2.1.4. Preparation of Short β-Lactoglobulin Rod-Like Aggregates. A solution of long β-lactoglobulin fibers, prepared as mentioned in section 2.1.2, was diluted to 1 wt % with pH 2 Milli-Q water and homogenized at 600 + 100 bar on five cycles using a two-stage high-pressure homogenizer (Panda 2K, Niro Soavi, Italy). This process enabled us to obtain shorter rod-like fibers from the long ones, while maintaining the same cross section. These short rods have an approximate diameter of 4 nm, and a contour length of 200 ± 100 nm,[34] which is well below the persistence length of the parent fibers.

From now on, we refer in this manuscript to this system as "processed short rods". This system is a mixed system of approximately 75% short rods and 25% unconverted monomers and partially hydrolyzed peptides.

2.1.5. Preparation of Pure Short Rods by Dialysis. A dialysis, as already described in section 2.1.3, was performed on the solution of 1% short processed rods, in order to obtain a pure system of short rods only. Note that this dialysis removes both unconverted monomers and the peptides. From now on, we refer in this manuscript to this system as "dialyzed short rods".

2.1.6. Summary of the Systems Investigated in This Work. All the systems investigated in this work are summarized in Table 2.

2.2. Methods. Otherwise stated, all the measurements were performed at 23 °C and were repeated at least three times. Average

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values and standard deviation values were extracted from these repetitions. The protein solutions were diluted to 0.1 wt % and were maintained at pH 2 for both surface tension measurements and interfacial rheology. For the dialyzed samples, the concentration of 0.1 wt % was achieved after the dialysis, in order to guarantee a direct quantitative comparison with processed samples.

2.2.1. Surface Tension Measurements at Air/Water and Water/Oil Interfaces. Protein adsorption at the water/air interface was measured using a Maximum Bubble Pressure Tensiometer (MPT2, Lauda, Germany) and a drop tensiometer (Tracker, I.T. Concept, France). The maximum bubble pressure tensiometry allowed access to a time range from 10^{-3} s to 10 s. A capillary of 0.075 mm radius was used to generate air bubbles in the protein solution with a decreasing flow rate from 120 to 2.5 $mm^3.s^{-1}$. A full description of the methodology can be found in the paper of Fainerman and Miller.[44] The drop tensiometer allowed access to time scales above 10 s, with continuous measurements of the rising drop area and volume, approximated by a Laplacian profile. The surface tension of a 12 mm^2 air bubble in a protein solution was followed for at least 16.7 h, i.e., the same time range used in the investigation of the interfacial complex modulus by interfacial shear rheology.

Interfacial tension at the water/oil interface was monitored using a drop tensiometer (Tracker, I.T. Concept, France) and the rising drop method. The aqueous phase was the protein solution, and the lipidic phase was MCT (medium chain triglycerides) oil (Delios, Cognis GmbH, Germany). The surface tension of a 12 mm^2 protein solution droplet in MCT oil was followed for 16.7 h.

2.2.2. Interfacial Shear Rheology at Water/MCT Oil Interface. Interfacial shear rheology measurements were performed using a Physica MCR500 rheometer (Anton Paar, Germany) and a bicone geometry (BI-68.5 type, 5° angle, 40 mm diameter) placed at the water/oil interface. The aqueous phase was the protein solution, and the lipidic phase was MCT oil. The setup for interfacial rheology and the procedures for the measurements are described in Erni et al.[45]

Before measuring, the following checks were performed: the horizontal level of the rheometer, the inertia of the device and the measuring system were all measured. Performing a motor adjustment prior to each measurement series allowed one to achieve a very low noise level, giving access to reliable measurements for torque values as low as 0.1 μN m.

All experiments were always carried out in the same order. The bicone was positioned at the water/MCT oil interface following the procedure described by Erni et al.[45] Approximately 3 min was needed to position the bicone at the water−air interface and cover the aqueous phase with oil. The recording of the data started approximately 1 min after the oil phase was layered on top of the aqueous phase. A small amplitude oscillation (strain 0.3%, frequency 0.1%) was applied and the interfacial complex modulus $|G_i^*|$ and the interfacial damping factor $\tan(\delta_i) = G_i''/G_i'$ were recorded versus time. The interface was allowed to age for 16.7 h. After this period, the evolution of $|G_i^*|$ was very slow: $d \ln|G_i^*|/dt$ was below 2.6% per hour, therefore, its increase over the experimental time of several hours did not exceed 10%. Finally, rheological experiments were conducted on this mature interface.

On the first sample, the linear domain limit was determined by a strain amplitude sweep from 0.1 to 10% at a constant frequency of 0.1 Hz. For the following experiments, a new sample was taken and a new interface was formed as previously described. Then, the following experiments were performed on the mature interface: (1) A frequency sweep was performed between 0.001 and 10 Hz, at a constant strain of 0.3%. For higher frequencies, large bulk dissipation and inertia effects were observed and the values for $f \geq 1$ Hz had to be discarded, by comparison with a blank reference sample without proteins at the interface. For some samples, the highest acceptable frequency was slightly less than 1 Hz. (2) A creep compliance test was performed in the linear domain, by applying a constant torque.[45] The torque imposed was always low enough so that the final interfacial deformation $D(t)$ was less than 2%. (3) To determine yielding, a constant shear rate of 0.01 s^{-1} was applied, until the strain reached between 100% and 1000%. The relevance of the experimental protocol and its limits will be discussed in the following parts of this manuscript. All experiments where strain was imposed were performed in the direct strain oscillation mode of the MCR500 instruments.

The value of the interface contribution to the bulk one, to the total torque value can be estimated through the Boussinesq number, Bo

$$\mathrm{Bo} = \frac{\eta_i}{(\eta_1 + \eta_2)R} \tag{1}$$

with η_i interfacial shear viscosity (Pa.s.m), η_1 and η_2 viscosities of the bulk phases (Pa.s), and R a characteristic distance of the flow geometry, in our case the diameter of the bicone (m).[45] The Boussinesq number can also be estimated during the formation of the interface probed in oscillatory mode, using eq 2:

$$\mathrm{Bo} = \frac{|G_i^*|}{(\eta_1 + \eta_2)\omega R} \tag{2}$$

with $|G_i^*|$ the interfacial complex modulus (Pa.m) and ω the pulsation (rad.s^{-1}).

If Bo $\gg$ 1, the contribution from the bulk can be considered negligible and the motion effects of the interface and of the bulk are not coupled. If Bo $\approx$ 1, the contributions from the bulk and the interface are of the same order of magnitude, with coupled motions. The determination of $|G_i^*|$ from the total torque measured then needs the solution of an implicit equation involving both interfacial and bulk contributions to the total stress.[45]

All bulk phases used in our work displayed Newtonian behavior in the windows of shear rates and frequencies investigated, for the given concentration of 0.1 wt % and pH 2. The viscosities of the aqueous phase were as follows: 1×10^{-3} Pa.s for the monomers, 1.5×10^{-3} Pa.s for the short rods, and 2.5×10^{-3} Pa.s for the long fibers. The viscosity of the MCT oil phase was 2.6×10^{-2} Pa.s. This justifies the use of the assumptions initially discussed by Oh and Slattery[46] for all experiments discussed here, and their adaptation to the oscillatory cases as well.[45] The interfacial parameters were thus extracted from raw data by performing an analysis described in Erni et al.,[45] using the *Rheoplus* software (Anton Paar, Germany).

2.2.3. Kjeldahl Assay. Deamidation is a chemical modification of the amide group of asparagine (Asn) or glutamine (Gln) residues, which can occur during a heat treatment. During deamidation, the amide functional group is replaced by a carboxylate group, releasing ammonia in the media. Therefore, quantification of the nitrogen content in the media before and after the heat treatment is a relevant method for estimating the extent of deamidation of β-lactoglobulin. Our objective was to assess whether the unconverted β-lactoglobulin monomers were chemically modified by the applied heat treatment.

A solution of heat-induced β-lactoglobulin fibers (2 wt %, pH 2) was filtered using a stirred ultrafiltration cell (Millipore, Bedford, MA) and an Omega 10 kDa membrane. The nitrogen content of the filtrate was determined using the Kjeldahl method and compared to the nitrogen content of the filtrate of a solution of native β-lactoglobulin monomers (2 wt %, pH 2) filtered in the same way. The determination of the nitrogen content was carried out using an automated Kjeldahl Unit (Auto Kjeldahl Unit K-370, Büchi, Switzerland) coupled with a sampler (Kjeldahl

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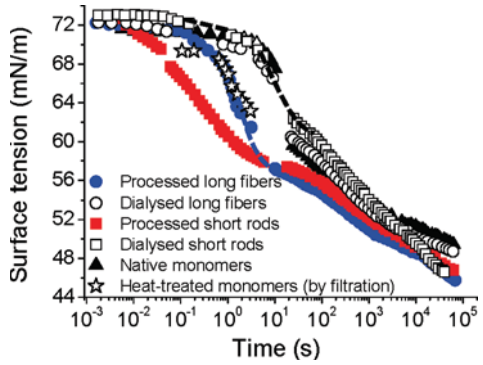

Figure 1. Surface tension of different β-lactoglobulin systems at the water/air interface measured using MPT2 and Tracker. The dashed lines were used as a guide to the eyes to link data from both techniques. Stars: surface tension of heat-treated monomers filtered from the processed long fibers.

sampler K-371, Büchi, Switzerland). For each sample, three measurements were carried out.

The nitrogen content was determined using eq 3

$$N_{\text{total}}(\%) = \frac{V_{\text{HCl}} \times C_{\text{HCl}} \times M_{\text{N}}}{10 \times V_{\text{tot}}} \qquad (3)$$

With V_{HCl} the volume of added HCl (in mL), C_{HCl} the concentration of HCl (in mol.g^{-1}), M_{N} the molecular weight of nitrogen (14 g·mol^{-1}), and V_{tot} the volume of the sample (in mL).

3. Results

3.1. Surface Tension of β-Lactoglobulin Systems. Figure 1 shows the evolution of surface tension of different β-lactoglobulin systems adsorbed at the water/air interface measured by MPT2 and Tracker at short and long times, respectively. The reproducibility was ±0.5% for MPT2 and ±5% for the Tracker.

As seen from Figure 1, the surface tension of all investigated β-lactoglobulin systems is globally decreased from ~72 mN/m to 45−52 mN/m after 16.7 h of adsorption (~6×10^4 s).

In the very short times investigated using MP2, the different β-lactoglobulin systems exhibit significantly different adsorption kinetics that can be divided into three groups. The first one is the processed short rods (red filled squares), for which surface tension decreases most rapidly and continuously. The second group is the processed long fibers (blue filled circles), for which the surface tension decreases at a slightly later stage in the beginning, yet with a greater slope at intermediate times, decreasing the surface tension from 70 to 56 mN/m in approximately 10 s. Finally, the third group contains the monomers (black filled triangles), the dialyzed long fibers (black open circles), and the dialyzed short rods (black open squares). For these three systems, the surface tension starts decreasing significantly only after several seconds.

The adsorption kinetics can be explained by comparing the processed systems with the dialyzed systems. Indeed, both dialyzed long fibers and dialyzed short rod systems show slower adsorption kinetics than the processed systems, having a behavior closer to the native monomer system. Therefore, the fast decrease observed for both processed systems can be attributed to the presence of small molecules, which are present in the processed systems but not in the dialyzed ones. These are monomers which did not aggregate into fibers during heat treatment. The adsorption kinetics of these unconverted monomers, obtained from ultrafiltration of processed long fibers, was measured by MPT2 and is also shown in Figure 1 (black open stars), giving two sets of information: (1) The slope of the curve in the range 0.3−3 s is very similar to that of the processed long rods, indicating that the fast decrease of surface tension in the 1−10 s range can be attributed to the presence of unconverted monomers. Indeed, dialyzed short and long fibers both exhibit totally different adsorption kinetics. (2) The curve is different from the one for the native monomers, showing that the unconverted monomers are different from native monomers. Moreover, because the concentration of these small molecules was estimated to be about 0.025%, due to the ultrafiltration process itself, they are also much more surface active than the native monomers. The attempt to reconstitute the processed long fiber system by mixing dialyzed long rods with native monomers at the proper ratio also confirmed this difference with the adsorption kinetics of this mixture being slower than the processed long fibers (not shown).

Table 3. Nitrogen Content of Filtered Solutions of β-Lactoglobulin before and after the Heat Treatment at pH 2

sample	total N concentration (%)
before the heat treatment	0.018%
after the heat treatment	0.023%

Analytical experiments performed on both the native β-lactoglobulin monomer solution and the unconverted monomers removed from the dialysis confirmed this hypothesis. The nitrogen contents determined using the Kjeldahl method are shown in Table 3.

The difference in the nitrogen content yields 0.005%, which represents 3.57 mM (M_{N} = 14 g·mol^{-1}). In one β-lactoglobulin monomer, there are 14 amino acids (5 Asn + 9 Gln), which can be deamidated out of the 162 total amino acids. In a 2 wt % solution, the molarity of β-lactoglobulin is 1 mM, whereas the molarity of all Asn and Gln residues is 14 mM. Therefore, with the ratio 3.57/14 ≈ 25%, we can conclude that approximately 25% of the 14 possible amino acids for deamidation are chemically modified during the applied heat treatment. This represents 3.5 amino acids per β-lactoglobulin monomer. Therefore, the heating protocol significantly affects the unconverted monomers, which, from a chemical point of view, differ from the native ones.

To investigate possible physical denaturation of the unconverted monomers after the heat treatment, the solution of heat-treated β-lactoglobulin was filtered through a 30 kDa membrane and analyzed by RP-HPLC and HP-SEC (see Supporting Information). For comparison, the native solution of β-lactoglobulin was also analyzed in the same experimental conditions. The filtering capacity of the 30 kDa membrane was previously estimated by filtering a solution of native β-lactoglobulin at 2% and pH 2 and determining the concentration of β-lactoglobulin in the filtrate by UV-spectroscopy at 278 nm: more than 90% of the initial monomers were retained by the membrane. RP-HPLC performed on the filtrate of the heat-treated β-lactoglobulin solution showed a population of peaks indicating the presence of various molecules with a higher hydrophobicity, while the characteristic peak for the native β-lactoglobulin monomer was no longer present. However, this can be attributed to the filtration step which retained most of the unconverted monomers. HP-SEC experiments performed in dissociating conditions on the filtrate of the heat-treated solution also showed a population of molecules having a molecular weight smaller than the β-lactoglobulin monomer. These are hydrolyzed peptides generated by the heat treatment. By calculating the ratio of the total areas for all the peptides over the total area for native β-lactoglobulin, we estimated that roughly 17% of the unconverted monomers were hydrolyzed into peptides after the heat treatment. Therefore, we can conclude that heat treatment induces extensive chemical and physical denaturation among the 25% unconverted monomers.

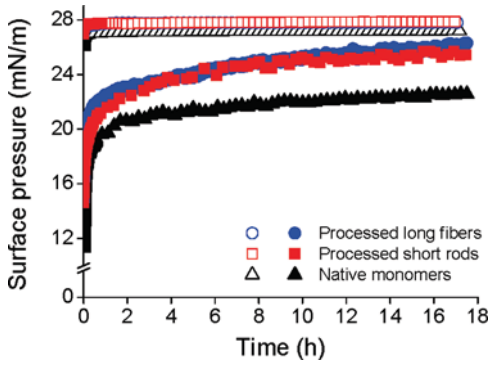

Figure 2. Comparison of surface pressure of different β-lactoglobulin systems at water/air (filled symbols) and water/MCT interfaces (open symbols).

The faster decrease of the surface tension observed for the processed short rods compared to the long fibers at short times (< 10 s) may find an explanation mainly in the homogenization process, which is applied to cut the long fibers into short rods. The shearing applied is likely to generate additional monomers coming from the fibers, as the solution goes through five cycles of homogenization. This hypothesis is supported by the fact that, once the short rods are dialyzed, their adsorption kinetics are slower and similar to that of processed long fibers, inferring that the presence of additional monomers and peptides is the main explanation for the faster decrease observed for the processed short rods.

After 16.7 h of adsorption, there is reduced difference among adsorption kinetics. Only the processed long fibers and short rods still have the smallest surface tension values. It is worth noting that surface tension is still decreasing for all systems; thus, no equilibrium or steady state seems to be reached yet after such a long time.

Interfacial activity of β-lactoglobulin systems was also investigated at the water/MCT interface. Figure 2 compares the evolution of surface pressure $\gamma_0-\gamma$ for the investigated β-lactoglobulin systems at both water/air and water/MCT interfaces, with γ the interfacial tension and γ_0 the interfacial tension in the absence of protein. At 25 °C, γ_0 is 72 mN/m for water/air interface and 30 mN/m for water/MCT interface.

As can be seen from Figure 2, the surface pressure at the water/MCT interface is higher than at the water/air interface, and it reaches a steady value within one hour, whereas at the water/air interface, surface pressure starts from lower values and continuously increases during the 16.7 h of adsorption. This comparison shows that the adsorption is faster at the water/MCT interface than at the water/air interface, in agreement with the work of Roth et al.[22] Proteins would absorb more rapidly at the water/oil interface due to a greater affinity of the hydrophobic patches for the oil phase rather than for air.

3.2. Interfacial Shear Rheology. *3.2.1. Interface Formation*. The linear viscoelastic properties of the interface were measured as a function of time during interface evolution of β-lactoglobulin systems, as shown in Figure 3A,B. The reproducibility upon repetition of the whole experimental protocol was $\pm 10\%$ for a given system.

As can be seen in Figure 3A, for all investigated systems, the interfacial modulus $|G_i^*|$ increases during the whole experiment, showing a continuous formation and strengthening of an interface over 16.7 h. For all β-lactoglobulin systems with fibers, the modulus increases very steeply from the beginning, whereas for the monomer system, a lag time can be observed and the modulus

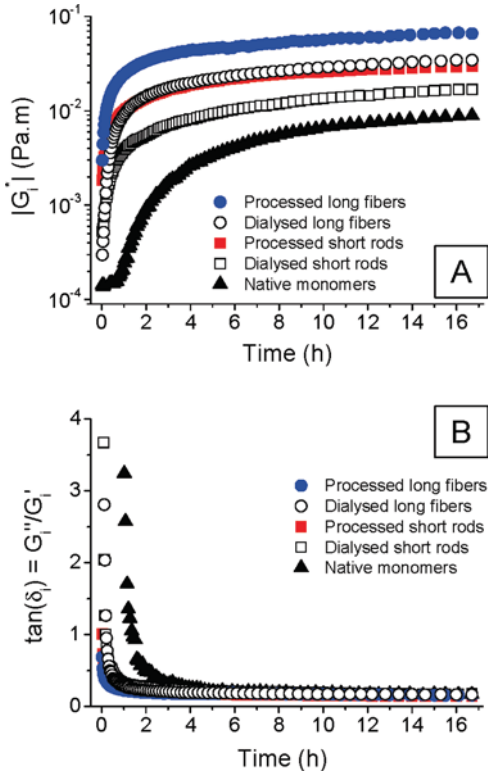

Figure 3. Evolution of the shear viscoelastic properties of the oil−water interface during absorption. Interface of different β-lactoglobulin systems in time as followed by (A) the shear interfacial complex modulus $|G_i^*|$ and (B) the interfacial damping factor $\tan(\delta_i)$.

starts increasing only one hour after the beginning of interface formation. The damping factor $\tan(\delta_i) = G_i''/G_i'$ gives insight into the viscoelastic properties of the interface during its formation (Figure 3B). For β-lactoglobulin systems with fibers, the interface remains mainly viscous within the first hour, then very quickly becomes mainly elastic. For the monomers, the interface becomes mainly elastic only after two hours of interface formation. After 16.7 h, $\tan(\delta_i) \approx 0.14-0.17$ for all systems. It can be clearly seen from Figure 3A that the long fibers (circles) build the strongest interface. The short rods (squares) have intermediate values of $|G_i^*|$ and the monomers (triangles) have the lowest $|G_i^*|$ modulus. After 16.7 h, the $|G_i^*|$ of the interface formed with the processed long fibers is 7−8 times stronger than the one built with the native monomers. The presence/absence of unconverted monomers and peptides in the systems with fibers also has a significant effect on the value of $|G_i^*|$. Processed systems have higher $|G_i^*|$ values than dialyzed systems, showing the significant contribution of the small molecules in the mixture with the fibers. The results shown in Figure 3 can be explained in terms of interactions between fibers and monomers adsorbed at the interface and will be discussed in section 4.

To estimate the respective contributions of the interface and the bulk during interface formation, the Boussinesq number Bo was calculated using eq 2. The evolution of Bo is the same as the evolution of $|G_i^*|$, as they only differ by a constant factor. Determining Bo over the entire interface formation period allowed assess of that for β-lactoglobulin systems with fibers; the contribution from the interface soon becomes dominant, whereas this happens for monomers only at a later stage. As a comparison, for systems with fibers, Bo < 1 for times below 10 min, whereas for the monomers, Bo < 1 for times below

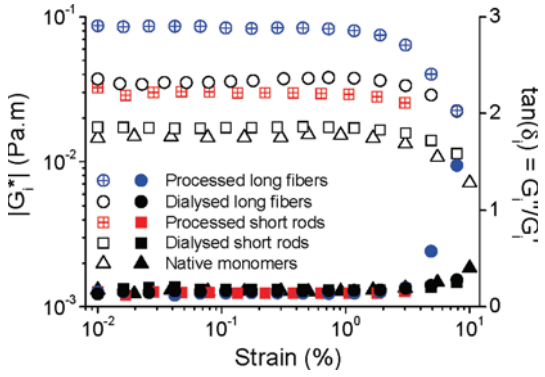

Figure 4. Strain amplitude applied to interfaces formed with different β-lactoglobulin systems. Open symbols: $|G_i^*|$. Filled symbols: $\tan(\delta_i)$.

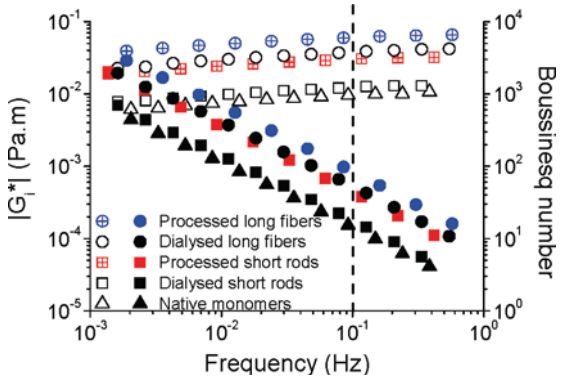

Figure 5. Frequency sweep applied to interfaces formed by different β-lactoglobulin systems. Open symbols: $|G_i^*|$. Filled symbols: Boussinesq number.

100 min. Bo < 10 below 50 min for systems with long fibers, below 3 h for systems with short rods, and below 10 h for the monomers. After 16.7 h from interface formation, Bo $\approx$ 90 for the processed long fibers, 50 for the dialyzed long fibers, 40 for the processed short rods, 25 for the dialyzed short rods, and 15 for the monomers. Taken together, all these data also show that what is measured after interface formation is mainly the interface and not the bulk contribution.

After 16.7 h of interface formation, the evolution of $|G_i^*|$ is slow enough to allow one to carry out all the following rheological experiments without having a significant contribution of the aging effect on the results. The variation of $|G_i^*|$ was less than 2.6% per hour during the whole time frame of the experiments performed after 16.7 h of interface formation.

3.2.2. Linear Domain. The linear domain limit of the interfaces formed by the β-lactoglobulin systems was investigated as shown in Figure 4.

For all investigated β-lactoglobulin systems, the linear domain limit lies between 1% and 2%. This is in agreement with literature, on lysozyme and β-casein layers, for which a limit of 2% was found.[47] Interestingly, despite different values for the interfacial complex modulus $|G_i^*|$, the interfacial damping factor $\tan(\delta_i)$ yields almost the same value in the linear domain. Above 1−2% strain, the drop of $|G_i^*|$ and the increase of $\tan(\delta_i)$ indicates nonlinear interfacial deformation, resulting in a change of the material properties of the interface, which becomes less elastic and more compliant.

3.2.3. Frequency Sweep. Figure 5 shows the results of the frequency sweep applied to interfaces formed with different β-lactoglobulin systems. Only the frequency range between 10^{-3} and 1 Hz is shown in the graph, since below 10^{-3} Hz, what is measured is mainly noise, whereas above 1 Hz, there is a major contribution from inertial effects, as indicated by the asymptotic $\sim\omega^2$ scaling of $|G_i^*|$ at the high-frequency limit. The inertial contribution for an aqueous−oily system was probed varying frequency at a given strain amplitude, with pure water instead of protein solutions. This gave a nearly quantitative characterization of their contribution, which in turn allowed discarding of all data points flawed by inertial contributions, resulting in the limited frequency window reported herein.

The interfacial modulus $|G_i^*|$ increases with the frequency, with the same slope for all the systems. The value of $\tan(\delta_i)$ (not shown) remained constant over the explored frequency range and was below 0.20 and very similar for all systems, implying that all the interfaces exhibit mainly elastic solid-like behavior at such frequencies. Together with the similar frequency dependence of the moduli of all systems, it suggests a similar and very broad spectrum of relaxation times of the dominant viscoelastic modes in the probed time scales, for all systems. The Boussinesq number Bo was calculated for the frequency range explored using eq 2 and plotted in Figure 5 (filled symbols). At low frequency values, the Boussinesq numbers achieved the highest values, from 600 to 3500, for the monomers and the long fibers, respectively. Bo decreases with increasing frequency and, at a frequency of 0.5 Hz, reaches a value between 3 and 16, for the monomers and the long fibers, respectively.

Note that the values of $|G_i^*|$ at 0.1 Hz are still comparable to the values measured right after the completion of interface formation of 16.7 h duration, shown in Figure 3.

3.2.4. Creep Compliance in the Linear Domain. Creep compliance experiments were performed on the β-lactoglobulin systems, applying an interfacial shear stress of 0.1 mPa.m (Figure 6A). The linearity of the interfacial deformation to the imposed interfacial shear stress was always verified over the whole range of deformations and times probed, including the first jump, if we disregard some distortions due to inertial effects upon startup.

The resulting deformation allows one to verify that the shear rate is small enough (smaller than 10^{-3} s^{-1}) to observe only the interfacial contribution in the total stress. The bulk stress being negligible, the results can be normalized by an interfacial stress σ, yielding the compliance function $J(t) = D(t)/\sigma$, where D is the interfacial deformation. The deformation always consists of one initial jump, corresponding to one or several very small relaxation times, i.e., an elastic part, followed by additional deformation over a large window of relaxation modes. One striking result from these experiments was the possibility of superimposing all compliance curves $J(t)$ in the experimental window probed, simply by using a single renormalization factor for the amplitude of $J(t)$ (Figure 6B). This remarkable result means that the aged systems only differ by their apparent modulus, regardless of the time scales considered, which we will discuss later in section 4.

Fitting of the creep curve for the processed long rods (blue filled circles in Figure 6) was done, using a power law $J(t) = J_0 + b.t^{\alpha}$, where J_0 is the fast elastic response (time 0), $b = (J_V/\tau^{\alpha})$, with τ the relaxation time, α an exponent between 0 and 1, and J_V the characteristic compliance of the viscoelastic part ($J(t) = J_0 + J_V$ for $t = \tau$). A fit with these 3 adjustable parameters worked very well, yielding $J_0 = 0.0139$ $Pa^{-1}.m^{-1}$, $b = 0.001\,59$ $Pa^{-1}.m^{-1}.s^{-\alpha}$, $\alpha = 0.37$ (not shown). It should be noticed that a power-law fit is

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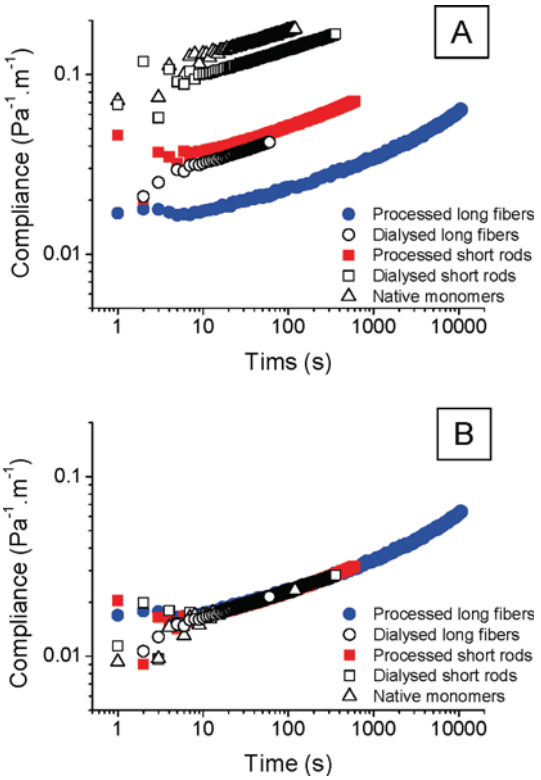

Figure 6. (A) Creep compliance on β-lactoglobulin systems where an interfacial shear stress of 0.1 mPa.m is applied; (B) Creep compliance curves of β-lactoglobulin shifted using a multiplying factor, showing a superimposition of all the curves. All curves were superimposed on the curve for the processed long fibers.

equivalent to a fit with a stretched exponential $J(t) = J_0 + J_V[1 - \exp(-(t/\tau)^{\alpha}])$,[48] that would reach a constant compliance value at infinitely long times, but with the choice of τ much larger than the experimental time. Hence, the power-law fit, which needs only 3 parameters and not 4, makes much more sense mathematically, and the fitting parameters are simply linked to the rheological ones, as shown above.

3.2.5. Nonlinear Behavior and Yielding. The mechanical response to a strain can be measured via the yielding experiment, as shown in Figure 7, where a constant macroscopic shear rate of 0.01 s^{-1} was applied to the systems in steady-rotation mode. It must be pointed out that the strain and the strain rate values are given for the whole interfacial gap only before yielding occurs. Indeed, yielding is accompanied by the appearance of domains and heterogeneous material properties at the interface.[49,50] Hence, the long-time interfacial stress values should not be interpreted as corresponding to an interfacial viscosity.

As seen in Figure 7, different responses are obtained from the systems investigated when they are being deformed. Values of the maximal shear stress response and the corresponding strain value are summarized in Table 4.

As seen from Table 4, the systems all have a different interfacial shear stress response and a different strain value. The interface is destroyed at the maximal interfacial shear stress, which decreases afterward. When the same deformation protocol is repeated a few minutes after the first yielding experiment, an interfacial shear stress peak can no longer be observed (data not shown).

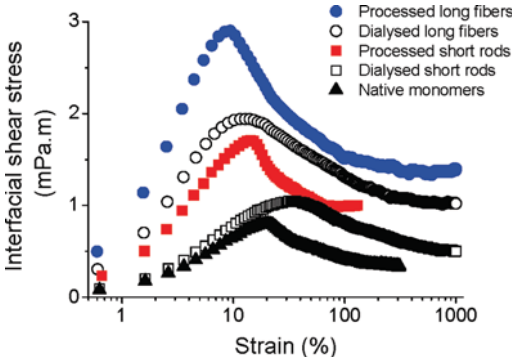

Figure 7. Interfacial yielding of different β-lactoglobulin systems, on which a constant macroscopic shear rate 0.01 s^{-1} is applied.

Table 4. Interfacial Yielding of Different β-Lactoglobulin Systems

systems	maximal interfacial shear stress	maximal strain
processed long fibers	2.9 Pa	9.4%
dialyzed long fibers	1.9 Pa	11.6%
processed short rods	1.7 Pa	14.3%
dialyzed short rods	1.1 Pa	33.6%
monomers	0.8 Pa	19.6%

The processed long fibers give the highest interfacial shear stress response, for the smallest maximal strain, and the interfacial shear stress value after deformation is the highest among the different systems. This indicates that their interface is the most rigid among the systems studied and the most fragile. For the dialyzed long fiber system, the interface is destroyed at a similar strain, but the shear stress peak is lower by a factor of two, showing the significant contribution of the unconverted monomers and peptides to the yield response of the interface. The dialyzed short rods show a similar behavior to the corresponding dialyzed long fibers, with a higher maximal strain and lower shear stress peak. In the presence of unconverted monomers and hydrolyzed peptides (processed short rods), the modulus is higher and the maximum strain lower. Particularly remarkable are the similarities of the curves among the processed fibers (both long and short) with respect to the dialyzed ones (both long and short). The stress−strain curve has a sharper peak in the former cases, whereas this is much broader in the case of dialyzed fibers. This indicates that, despite the plateau value in stress, which is obviously higher when the fibers are longer, the rigidity of the interface is highly dependent on the presence of small molecules such as unconverted monomers. Finally, the interface stabilized by monomers only can deform up to 20% before being destroyed and the shear interfacial stress response is less than 1 mPa.m, implying that this is the least rigid interface against deformation among the systems investigated.

4. Discussion

The kinetics and mechanisms of adsorption of β-lactoglobulin have been studied extensively in the literature for pH values from 4 to 9. The adsorption behavior of β-lactoglobulin has not been thoroughly characterized at pH 2, and heat-induced β-lactoglobulin fibers have not been investigated yet. Their study represents a challenging task, due to the complexity of the system. The dialyzed fibers (long and short) constitute a more model system, in the sense that the contribution of the small molecules coexisting with the processed fibers can be neglected. Therefore, direct

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comparison of these systems with the processed ones can give insight into the contribution of each individual compound during the adsorption process.

Concerning the interfacial tension measurements, the difference between the adsorption kinetics at the water−MCT interface and the water−air interface is clear and expected.[14] At the water−MCT interface, the surface pressure values reach a plateau within 1 h of adsorption, with a value close to 28 mN/m for all systems (Figure 2). Starting from an initial protein bulk concentration of 0.1 wt %, the effect of the adsorbed amount on the average bulk concentration can be considered negligible; therefore, the chemical potential of the proteins in the bulk remains constant. Since no significant differences in the adsorption kinetics at the water−MCT interface can be found for the investigated systems, the interfacial shear rheology data will be discussed independently from the interfacial tension results.

The literature concerning β-lactoglobulin-adsorbed interfaces discusses quite extensively results for pH values above 4,[24,32,33] but much less can be found when it comes to pH 2.[22] Still, remarkable trends described in the literature as a function of the concentration and the pH are worth being recalled here. First, it appears that the initial bulk concentration has a direct effect on the adsorption behavior of β-lactoglobulin: at very low bulk concentrations (10^{-5} to 10^{-4} wt %), the adsorption reaches an interfacial saturation value which is far below the monolayer saturation value, but with a fast kinetics (300 s).[24,33,51] Effects on the interactions in the vicinity of the interface that may be specific to low concentrations are discussed in Donsmark and Rischel's work.[24] At a higher bulk concentration, the reached saturation value is usually an order of magnitude higher, and a plateau is reached within several hours.[14]

Second, the pH has a significant effect on the surface density of adsorbed proteins, as it determines the protein net charge. Together with ionic strength, pH therefore influences the extent of electrostatic or ionic interactions. In the literature, Donsmark et al. showed that changing the pH from 6 to 9 (which corresponds approximately to a change from −7 to −12 for the protein net charge[52]) decreased the surface density of β-lactoglobulin-adsorbed interface from a low initial bulk concentration, by a factor of 2.[24] Since the net charge at pH 2 is about twice as high as the net charge at pH 7,[52] it is worth considering, at least qualitatively, what is described in the literature in that respect.

If we have a general overview of the interfacial shear rheology results, it can be seen that the investigated systems all build a viscoelastic interface within a few hours, which soon acquires a dominant, solid elastic behavior in the frequency range 10^{-3}−1 Hz. However, the long-term creep compliance responses suggest that the systems appear as such because of a very broad spectrum of relaxation times, with a contribution of relaxation times that could be longer than a day. Let us now analyze the interfacial rheology data in more detail, where differences between the systems become visible.

First, an interface of adsorbed β-lactoglobulin was formed by leaving the adsorption and aging to proceed (Figure 3A). The interface was left to form for 16.7 h under small oscillations (frequency 0.1 Hz, strain 0.3%). After that, it was considered that the interface was mature and its evolution could be neglected during the following experiments. At this stage, the systems already display significant differences in interfacial moduli. The interface of native β-lactoglobulin monomers displays the lowest interfacial modulus among the investigated systems (8.9 mPa.m). The interface of dialyzed short rods has a slightly higher modulus (16.9 mPa.m), and the interface of dialyzed long fibers has a higher modulus (34.5 mPa.m). Obviously, from our results, the aspect ratio is not the only parameter accounting for interfacial modulus.[53] The flexibility and the polydispersity of the long fibers are probably two other important parameters to take into account to explain the interfacial modulus. Comparing the interfacial moduli of interfaces of processed and dialyzed fibers, it is clearly seen that the processed system displays a modulus which is higher by a factor of approximately 2 compared to the dialyzed system. Therefore, the presence of unconverted monomers in the processed system, which amounts to 25%, significantly contributes to strengthening the interfacial modulus. This effect may be explained by a "fill-in" process of the unconverted monomers among the gaps created by the fibers during adsorption. It is to be expected that the polydispersity of the long fibers further contributes to enhancing their packing at the interface, and thus strengthening the interface.

The mature interfaces display differences in their shear moduli. However, there are great similarities between the systems, which we describe in detail hereafter. First, all the interfaces show a dominant elastic behavior (Figure 3B), after 1 h for the fiber systems and 3 h for the native β-lactoglobulin. Second, we see a low frequency dependence of the interfacial moduli for all the curves, which also have the same slope, regardless of their absolute value (Figure 5). This result, together with the fact that the value of $\tan(\delta_i)$ is almost the same for all the systems and below 0.20 over the explored frequency range, shows that all the interfaces have a solid elastic behavior and similar spectra of relaxation times. This is convincingly confirmed by the observation that the creep compliance curves can all be superimposed simply by applying a constant factor for each system (Figure 6B). This observation implies that the systems share the same ratio between the short-time elastic response (J_0) and the long-time viscoelastic response (included in the parameter b), within the experimental time window probed. These three experiments obviously show that some effects, for example, the contribution of the unconverted monomers on the interfacial modulus of processed systems, are not seen specifically in the linear regime. Therefore, the present results suggest that there is a fundamental type of interaction that leads to the observed similarity for all investigated systems.

From the literature, it is known that adsorbed β-lactoglobulin monomers at pH 2 are physically interacting through noncovalent bonds.[22] There are no chemical interactions, as the free thiol group of the cystein residue is not active at acidic pH and prevents any cross-linking through disulfide bonds. This was demonstrated with a competitive displacement experiment of β-lactoglobulin adsorbed at the oil−water interface by Tween 20, a non-ionic surfactant:[22] at pH 2, β-lactoglobulin was easily and totally displaced by Tween 20, with a much lower required concentration than at pH 7 and pH 5.6, respectively. This allows the conclusion that, at pH 2, the interactions are purely physical, unlike at pH 7 where chemical bonds occur, and moreover, they are mainly repulsive, unlike at pH 5.6 where hydrophobic interactions drive extensive aggregation upon adsorption. In addition, they also showed that heat-treated β-lactoglobulin at pH 2 was totally and easily displaced by Tween 20, whereas heat-treated β-lactoglobulin

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at pH 9, for which polymerization occurred, was not totally displaced, even with a high concentration of Tween 20.

Therefore, it can be expected that heat-induced β-lactoglobulin fibers will also interact through physical interactions at pH 2, which are likely to be mainly electrostatic repulsive interactions. Indeed, at this pH, β-lactoglobulin is highly positively charged; therefore, the long-range repulsive electrostatic interactions are believed to be the main driving interactions in the present case, leading possibly to a physical, reversible gel. In support of this hypothesis, Donsmark and Rischel have shown that the diffusion of β-lactoglobulin at the interface can be significantly slowed down due to interactions between proteins of dominantly electrostatic repulsive nature.[24] This view is supported by Cicuta et al.,[31,32] who showed the development of a shear modulus at a high surface pressure ($\sim$0.8 mg/m^2) and very slow relaxation dynamics, which both could be explained by a kinetic arrest due to jamming of β-lactoglobulin interacting dominantly via repulsive interactions.[31] As a consequence of the above arguments, it is highly likely that the dominating types of interactions for our systems at pH 2 are of electrostatic repulsive nature due to the high electric charges carried by both the β-lactoglobulin monomers and fibers. Additional experiments probing the interface with spectroscopic methods and their evolution and reversibility upon compression/decompression or competitive displacement experiments should allow confirmation of the nature of the interactions.

Finally, the last interfacial rheology experiment performed on the protein-adsorbed interfaces investigates their yielding behavior in the nonlinear domain (Figure 7). Unlike the previous results seen in the linear domain, here each interface responds differently to the applied macroscopic shear rate. First, it is observed that a higher modulus always gives a higher yield stress. Second, the interfaces formed with fibers having a contour length higher than the persistence length have the lowest yield strain. Third, the presence of unconverted monomers has a clear effect on the yielding of the interface, by increasing the interfacial shear stress response and decreasing the yield strain, and making the yielding transition sharper in the stress−strain curves. We can make a parallel with the interface formation in the linear domain (Figure 3), where the presence of unconverted monomers also had an effect on the rheological properties of the interface, by increasing the interfacial modulus. These two experiments show that, in the presence of unconverted monomers, the formed interface is stronger than without the unconverted monomers, and it shows a higher rigidity against deformation; however, it appears to be more fragile. These properties related to the presence/absence of unconverted monomers were not seen in the experiments carried out in the linear domain after interface formation; therefore, we may conclude that they are directly correlated to the interfacial modulus of the interfaces after 16.7 h of adsorption.

5. Conclusion

In this work, we have studied the interfacial properties of different types of β-lactoglobulin systems at pH 2, ranging from the native monomer to the heat-induced (long) semiflexible or (short) rigid aggregates and their dialyzed counterparts. These were investigated through interfacial tension measurements and interfacial shear rheology at the water−oil interface.

From the surface tension measurements at the water−air interface, we were able to shed light on two major phenomena. First, the fastest adsorption kinetics observed with the processed long and short fibers, especially in the short times (10^{-3}−60 s), are due to the presence of unconverted monomers and hydrolyzed peptides in mixture with the fibers. Second, both the dialyzed short and long fibers showed similar adsorption kinetics, which therefore seem to be independent from length or flexibility. Interfacial tension measurements at the water−oil interface showed significantly faster adsorption kinetics, with a surface pressure value reaching a plateau after 1 h of adsorption for all the systems.

Interfacial shear rheology experiments were performed on protein-adsorbed interfaces previously left to age for 16.7 h. All the β-lactoglobulin systems were able to form interfaces with a high interfacial modulus, the highest modulus being achieved with the processed long fibers and the lowest one with the native β-lactoglobulin. The presence of unconverted monomers significantly increased the interfacial modulus, compared to the dialyzed systems from which they were removed. However, despite the difference in the absolute values of interfacial modulus, the rheological experiments performed in the linear domain highlighted strong qualitative similarities in the viscoelastic behavior of all the interfaces: during interface formation, all interfaces rapidly developed an almost purely elastic behavior, which showed the same frequency dependence and the same spectrum of relaxation times. In contrast, the yielding experiment showed different responses depending on the system, with the highest interfacial yield stress response for the processed long fibers and the lowest one for the native β-lactoglobulin. Also, in the presence of monomers, the maximum affordable deformation before yield was reduced, indicating that the increase in rigidity is accompanied by a more fragile interfacial behavior. The presence/absence of unconverted monomers clearly had an effect on the rheological behavior of the interface, as during interface formation. These remarkable features obviously deserve further investigation, especially on the interactions between the proteins at the interface. Regarding the low pH used here, the dominant interactions between adsorbed proteins in the systems investigated are likely to be essentially of electrostatic repulsive nature and are inferred to be responsible for jamming at the interface, resulting in the solid-like behavior at observed time scales ($1-10^3$ s).

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