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Protein−Peptide Interaction: Study of Heat-Induced Aggregation and Gelation of β-Lactoglobulin in the Presence of Two Peptides from Its Own Hydrolysate

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ABSTRACT: Two peptides, [f135−158] and [f135−162]-SH, were used to study the binding of the peptides to native β-lactolobulin, as well as the subsequent effects on aggregation and gelation of β-lactoglobulin. The binding of the peptide [f135−158] to β-lactoglobulin at room temperature was confirmed by SELDI-TOF-MS. It was further illustrated by increased turbidity of mixed solutions of peptide and protein (at pH 7), indicating association of proteins and peptides in larger complexes. At pH below the isoelectric point of the protein, the presence of peptides did not lead to an increased turbidity, showing the absence of complexation. The protein−peptide complexes formed at pH 7 were found to dissociate directly upon heating. After prolonged heating, extensive aggregation was observed, whereas no aggregation was seen for the pure protein or pure peptide solutions. The presence of the free sulfhydryl group in [f135−162]-SH resulted in a 10 times increase in the amount of aggregation of β-lactoglobulin upon heating, illustrating the additional effect of the free sulfhydryl group. Subsequent studies on the gel strength of heat-induced gels also showed a clear difference between these two peptides. The replacement of additional β-lactoglobulin by [f135−158] resulted in a decrease in gel strength, whereas replacement by peptide [f135−162]-SH increased gel strength.

KEYWORDS: *protein−peptide interaction, β-lactoglobulin, aggregation, gelation*

INTRODUCTION

In several studies it has been shown that the aggregation and gelation behavior of proteins is affected by enzymatic hydrolysis. In all cases significant changes in the rheological properties and the appearance of gels were found.[1−5] Depending on the state of protein (native or denatured), enzyme used, the degree of hydrolysis, and the conditions of gelation, either an increased or a decreased gel strength was observed. Control over gel properties can thus be obtained by controlling the hydrolysate properties, although at this point it is not clear which properties the hydrolysate should have. It was further observed that under some conditions aggregation and gelation started during hydrolysis.[1,6,7] This shows that the peptides formed can associate to form aggregates. If the peptides can interact with each other, they might also interact with intact protein. From these studies the idea was formed that the addition of hydrolysates to intact protein solutions could be used to change the heat-induced aggregation and gelation of intact protein.

Previous studies indeed showed that the addition of whey protein isolate hydrolysate to a solution of intact whey protein isolate (WPI) induced protein aggregation at room temperature.[8] In another study, it was shown that the amount of intact β-lactoglobulin aggregates formed upon heating was increased by the presence of a soluble fraction of a β-lactoglobulin hydrolysate.[9] In the same study, the binding of peptides from the soluble β-lactoglobulin hydrolysate to intact β-lactoglobulin was studied by SELDI-TOF-MS. It was shown that 5 from the 28 peptides detected in the hydrolysate bound to the intact protein by noncovalent interactions.[10] Consequently, it was hypothesized that the binding of certain specific peptides to the intact proteins determines the observed effects on (heat-induced) aggregation and gelation. Because many peptides are present in the hydrolysate, it is difficult to find a direct relationship of the observation of the binding certain peptides in one experiment and effects on heat-induced aggregation in another. Therefore, in the present study, the effects of peptides on aggregation and gelation of intact β-lactoglobulin are studied using two of the β-lactoglobulin-derived peptides that were found to bind to β-lactoglobulin, [f135−158] and [f135−162]-SH. The peptide β-lactoglobulin [f135−158] was selected because it was previously found to induce and take part in protein aggregation during β-lactoglobulin hydrolysis by *Bacillus licheniformis* Protease (BLP).[8,11] Furthermore, it was found to bind to β-lactoglobulin and to be present as one of the dominant peptides in a heat-induced pellet of a mixture of β-lactoglobulin and its soluble BLP hydrolysate.[10] The other peptide, β-lactoglobulin [f135−162]-SH, is chosen because it is highly similar to the first peptide, except that it contains one free cysteine residue. The presence of this sulfhydryl group could lead to an additional effect. This cysteine residue could react with disulfide bridges in the intact protein, thereby affecting the gel strength. Broersen et al.[12] found that the

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Table 1. Characteristics of the Proteins and Peptides Used in This Study

name	exptl mass (Da)	theor mass (Da)	$H\Phi_{total}/[H\Phi_{av}]^a$ (kcal/[kcal × res^{-1}])	net charge at pH 7.0	p*I*	no. of SS/[SH]
[f135−158]	2827.0	2827.3	31.6 [1.31]	+1.1	8.5	0 [0]
[f135−162]-SH	3308.0	3308.9	35.5 [1.27]	+1.1	8.5	0 [1]
[f135−162]-SX	3433.0	3434.0	unknown	+1.1	8.5	0 [0]
[f159−162]-SH	499.6	499.3	4.0 [0.99]	0	7.2	0 [1]
β-lactoglobulin	nd[b]	18306	196 [1.21]	−8.3	5.2	2 [1]
ovalbumin	nd	42750	431 [1.12]	−11.6	5.2	1 [4]
lysozyme	nd	14313	127 [0.98]	+7.7	9.3	4 [0]

[a]Total and average hydrophobicity was calculated according to the method of Bigelow.[31] [b]Not determined.

introduction of four to six free thiol groups on ovalbumin resulted in an increase of branched microstructures and an increase of gel strength of >3 times. In another study, blocking all free sulfhydryl groups in WPI aggregates decreased the gel hardness after cold gelation to 10% of the initial hardness.[13] In the current study, the rheological properties of heat-induced gels are studied using the electrowetting technique.[14] In this technique, smaller sample volumes are needed as compared to traditional Couette rheometry (3 and 1000 μL respectively).

MATERIALS AND METHODS

β-Lactoglobulin was isolated from fresh bovine milk, purified, and characterized as described previously.[10] Lysozyme, L-cysteine, and *N*-ethylmaleimide (NEM) were obtained from Sigma (St. Louis, MO, USA), and ovalbumin was purified as described previously.[15] Three peptides based on sequences [f135−158], [f135−162]-SH, and [f159−162]-SH from *β*-lactoglobulin were chemically synthesized by Biomatik (Wilmington, DE, USA). The main characteristics of these peptides are given in Table 1. The purity of each peptide was found to be >95%, based on the RP-UPLC peak area at 214 nm compared to the total area (data not shown). Silicon oil SIL 180 (Thermo Fischer Scientific, Waltham, MA, USA) was filtered over a 0.22 μm filter (Schleicher & Schuell, Dassel, Germany) before use. *α*-Cyanohydroxycinnaminic acid (CHCA) was obtained from Bruker Daltonics (Bremen, Germany). All other chemicals were of analytical grade.

Blocking of Reactive Sulfhydryl Group. The reactive sulfhydryl group in peptide [f135−162]-SH was blocked by addition of 1.5 mL of a 10 mM solution of NEM in Milli-Q water to 1.5 mL of a 20 mg/mL (6 mM) peptide [f135−162]-SH solution.[13] The mixture was adjusted to pH 7.0 with 0.1 M NaOH and incubated for 3 h at room temperature. After incubation, the excess of NEM was removed by dialysis using a Float-A-Lyzer G2 membrane with a cutoff of 500−1000 Da (Spectrum Laboratories, Rancho Dominquez, CA, USA) overnight at 4 °C. The blocked peptide was subsequently freeze-dried. Blocking of the free sulfhydryl group was confirmed by the Ellman's reagent assay as described previously.[15] The modified peptide [f135−162]-SH is further denoted [f135−162]-SX.

Solubility Profiles. The solubility of *β*-lactoglobulin in the presence and absence of peptides [f135−158] and [f135−162]-SH was studied at pH 3−11. The peptides were dissolved in water at 5.0 mg/mL at pH 3 in the presence or absence of 5.0 mg/mL *β*-lactoglobulin. The pH was adjusted by the addition of 0.5 M NaOH to 1 mL of the solution in a quartz cuvette, after which the pH was measured. The transmission of the sample mixtures at 600 nm was used as a measure for the solubility and corrected for the dilution due to the amount of NaOH added. All measurements were carried out in duplicate

Circular Dichroism Spectroscopy. Far-UV circular dichroism experiments were carried out as described previously.[15] Typically, 1.0 mg/mL of peptide was dissolved in 50 mM sodium phosphate buffer, pH 7.0. For far-UV experiments the samples were diluted 10 times in the same buffer prior to the CD experiments, and spectra were recorded from 195 to 260 nm at 20 and 90 °C (total unfolding). The spectra were analyzed for secondary structure elements using the standard CDNN program,[16] using 33 reference spectra from structurally well-characterized globular proteins.

Surface-Enhanced Laser Desorption Ionization Time of Flight Mass Spectrometry (SELDI-TOF-MS). SELDI-TOF-MS experiments were performed as described earlier.[10] *β*-Lactoglobulin, ovalbumin, and lysozyme were covalently bound to separate spots on the ProteinChip PS-20. A fourth spot was left empty (but blocked with ethanolamine) to determine the nonspecific binding of peptides to the ProteinChip. Experiments were carried out in duplicate. The peptides were dissolved to a final concentration of 5.0 mg/mL in phosphate-buffered saline solution (PBS buffer) containing 0.1% (v/v) Triton X-100 (to reduce nonspecific binding), and the pH was adjusted to pH 7.4 by adding 0.1 M HCl or 0.1 M NaOH. Aliquots (5 μL) of this solution were added to the ProteinChip PS-20 surface and incubated in a humidity chamber overnight at 4 °C. After incubation, the ProteinChip PS-20 was subsequently washed three times by incubation for 10 min in an excess of PBS buffer, pH 7.4, containing 0.1% (v/v) Triton X-100. The ProteinChip PS-20 was washed in excess PBS buffer, pH 7.4, and in Milli-Q water prior to being air-dried. Before the MS experiment, 0.5 μL of CHCA solution was added, air-dried, and repeated again. The ProteinChip PS-20 was read at a laser intensity of 1 kAU.

Dynamic Light Scattering (DLS). DLS measurements were performed using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) equipped with a 4 mW HeNe laser beam with a wavelength of 633 nm, at a scattering angle of 173°. The intensity of scattered light (I_s) and the hydrodynamic diameter (d_h) of the monomers, peptides,and aggregates were measured for a solution of 5.0 mg/mL *β*-lactoglobulin in 50 mM sodium phosphate buffer, pH 7.0, containing 0, 0.1, 0.5, 1.0, 2.5, and 5.0 mg/mL peptides. These concentrations correspond to the molar protein/peptide ratios denoted in Table 2.

Table 2. Calculated Molar Protein/Peptide Ratios Based on 5.0 mg/mL *β*-Lactoglobulin Solution and Different Concentrations of Peptide Solution

	molar ratio protein/peptide		
concentration of peptide (mg/mL)	[f135−158]	[f135−162]-SH	[f135−162]-SX
0	0	0	0
0.1	10:1	10:1	10:1
0.5	10:7	10:6	10:5
1.0	10:13	10:11	10:11
2.5	10:32	10:28	10:27
5.0	10:66	10:56	10:53

Typically, 50 μL of sample was covered with 50 μL of paraffin oil to prevent water evaporation from the sample and analyzed for 130 or 300 s at 20 °C. Subsequently, the sample was removed from the Zetasizer Nano, and the temperature was set to 80 °C. When the Zetasizer Nano had reached the temperature set, the sample was placed back into the Zetasizer Nano and analyzed for at least 25 min. All measurements were carried out in duplicate.

Surface Tension Measurements. The interfacial tension at 20 °C between 50 mM sodium phosphate buffer, pH 7.0, and the silicone

oil was determined using an Automated Drop Tensiometer (ADT; I.T. Concept, Longessaigne, France). A pendant droplet of 7 μL containing the buffer was formed at the tip of a syringe needle placed in a cuvette containing the silicon oil. The surface tension, γ (N/m), was calculated by the software of I.T. Concept. This value was used in the calculation of the thickness of the dielectric support used in the electrowetting (EW) experiments.

Electrowetting. *Preparation of the Dielectric Support.* Dielectric supports were prepared as described previously.[14] A glass slide with a conductive indium tin oxide (ITO) layer was cleaned with Milli-Q water, 70% (v/v) ethanol, and *n*-heptane consecutively for 15 min in an ultrasonic bath for each step. Subsequently, the glass slide was dip-coated into a 6% (v/v) amorphous Teflon AF (1600) in perfluorinated solvent FC 75 (DuPont, Wilmington, DE, USA), retracted from this solvent at a speed of 15 cm/min, and dried for 10 min at 110 °C in a vacuum. The dip-coat and drying step were repeated once, and finally the support was dried first at 160 °C for 10 min and then at 340 °C for 30 min (again in a vacuum).

Preparation of Gelled Droplets. For the first experiments stock solutions of 120 mg/mL β-lactoglobulin in 50 mM sodium phosphate buffer, pH 7.0, were prepared in the presence or absence of 2.5 mg/mL peptide or 2.5 mg/mL β-lactoglobulin in the case of the blank. The stock solution was further diluted to reach concentrations ranging from 4 to 120 mg/mL β-lactoglobulin solution in the same buffer solution. In this way the total concentration was varied while the protein/peptide mass ratio of 48:1 is constant. For the second experiment the peptide concentration was varied. To this purpose, a β-lactoglobulin solution in 50 mM sodium phosphate buffer, pH 7.0, was mixed with the peptide solution to final peptide concentrations ranging from 0 to 50 mg/mL in 100 mg/mL β-lactoglobulin solution. For the blank, β-lactoglobulin was added to the β-lactoglobulin solution in the same concentrations. Samples were degassed for 10 min. From each solution several gelled droplets were made by adding a droplet of 3 μL to a Teflon vial containing 1 mL of silicone oil. Samples were heated for 45 min in a preheated oven at 80 °C. After heating, the samples were allowed to cool for 15 min at room temperature. The droplets were transferred from the Teflon vial onto the dielectric support, which was already placed in a glass container containing 10 mL of silicone oil at room temperature.

Microrheology. The complex modulus of heated protein solutions was determined using an EW setup (Figure 1).[17] An electrode

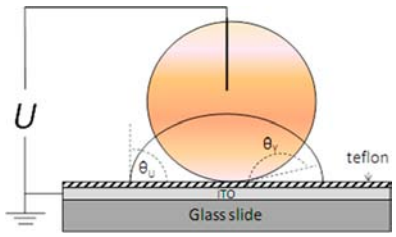

Figure 1. Schematic drawing of the electrowetting setup (U = voltage).

(Wolfram wire sharpened by electrolysis) was inserted in the top of the droplet. An oscillating voltage was then applied over the electrode in the droplet and the electrode in the dielectric support. The AC voltage (sine wave, frequency f_c = 1 kHz) was generated by a function generator (Hewlett-Packard 33120A, Palo Alto, CA, USA). The peak−peak voltage was varied between 0 and 30 V (yielding a root-mean-square voltage between 0 and 21.21 V) using amplitude modulation with a sinusoidal wave with a frequency of 10 mHz, The contact angle (cos θ) as a function of applied voltage (U) was monitored by an optical contact angle measuring system G10 (Krüss, Hamburg, Germany) and calculated by Drop Shape Analysis (DSA) software, version 1.90.0.14 (Krüss). Each droplet was measured during two time periods (200 s) of the amplitude modulation, at two different places on the support. In this way, at least four sets of data were obtained for each sample.

Calculations. To determine the elastic modulus of the gelled droplets, first, the thickness of the Teflon layer on the dielectric support was determined. For this determination, the change in contact angle [θ (rad)] of a 50 mM sodium phosphate buffer droplet, pH 7.0, was measured as a function of the applied voltage [U (V)]. The layer thickness was inferred from the classic EW equation[18]

$$\eta \equiv \frac{\varepsilon\varepsilon_0}{2d\gamma} \times U^2 = \cos(\theta) - \cos(\theta_Y) \quad (1)$$

where η is the dimensionless EW number, θ is the measured contact angle in the presence of the electric field, and θ_Y is Young's angle (contact angle in the absence of the electric field). Furthermore, ε = 2.1 is the relative dielectric permittivity of the Teflon layer, ε_0 the dielectric permittivity of vacuum, d the Teflon layer thickness (m), and γ the interfacial tension (N/m) of the buffer solution in silicone oil at 20 °C, which was determined independently and found to be equal to 1.34×10^{-2} N/m.

Electrowetting of nonheated protein droplets and nongelled heated protein droplets can also be analyzed with the traditional EW equation (eq 1). For a known thickness d of the Teflon layer, such an analysis then gives the surface tension of the protein-coated oil−buffer interface. For gelled droplets, an extension (eq 2) of the EW equation has been worked out by Banpurkar[14]

$$\eta = \cos(\theta) - \cos(\theta_Y) + \frac{8GR_0}{3\gamma\pi} \times H(\cos(\theta), \cos(\theta_Y)) \quad (2)$$

with

$$H(X, Y) = \left(\frac{4^{1\backslash 6}(3 + X)(1 + X)^{3/2}}{(1 - X)^{4/3}(2 + X)^{1/6}}\right) \times \left(1 - \frac{(1 - X)(2 + X)^{1/2}(1 + Y)^{3/2}}{(1 - Y)(2 + Y)^{1/2}(1 + X)^{3/2}}\right) \quad (3)$$

and R_0 the radius of the drop in the freely suspended state. To determine the complex shear modulus of gelled droplets at higher concentrations, a value for the surface tension was estimated from low concentration EW data (concentrations < 4% w/v). The surface tension value found from the EW data is independent of protein/peptide concentration for concentrations <4% (w/v). Hence, it is assumed that this value is similar to that of higher protein/peptide concentrations. This leaves the complex modulus G as the only adjustable parameter when using eq 3 to fit data at higher protein/peptide concentrations. For each sample, the complex modulus was calculated from the average obtained from two droplets that were all measured at two different locations of the substrate, in other words, as the average over four determinations. It has been shown that the results obtained for the complex modulus with this technique are comparable to those obtained with traditional bulk rheology.[14]

RESULTS AND DISCUSSION

Secondary Structure of Peptides. The relative amounts of secondary structure elements in the model peptides as determined by circular dichroism are listed in Table 3. The peptides contain 46−47% β-sheet structure and 48−49% random coil at 20 °C. α-Helix and β-turn structures are almost

Table 3. Relative Amount of Secondary Structure Elements (Percent ± 5%) of the Peptides at 20 and 80°C

	[f135−158]		[f135−162]-SH		[f135−162]-SX	
	20 °C	80 °C	20 °C	80 °C	20 °C	80 °C
α-helix	2	3	2	3	2	3
β-sheet	46	46	47	48	46	48
β-turn	3	5	3	4	3	4
random coil	49	46	48	45	49	45

negligible (2−4%). These values are in fairly good agreement with the literature about the presence of secondary structure in a mixture of related peptides,[9] but in contrast to the secondary structure of this sequence within the intact β-lactoglobulin molecule. Estimation of the amounts of structure elements by the software program RasMol (v2.7.5, RasWin Molecular Graphics, Bernstein & Sons, Bellport, NY, USA) reveals that the amino acid sequence 135−158/162 within β-lactoglobulin consists of about 30% α-helix and 15% β-sheet structure. Heating of the peptide solution at 80 °C for 10 min does not change the composition of secondary structure significantly. In our previous work[9] the secondary structure of β-lactoglobulin in the presence of a hydrolysate, containing a range of different peptides, was studied. It was shown that both at 20 °C and at 80 °C the structure of the protein in the presence of the hydrolysate was similar to that of the protein in the absence of the hydrolysate.

Binding of Peptides at Room Temperature. Interaction between intact protein and peptides was determined by the turbidity of β-lactoglobulin solutions in the pH range from 2 to 11, in the presence or absence of [f135−158] and [f135−162]-SH. Although the value for transmission in the case of pure β-lactoglobulin solution drops to approximately 40% at pH 5, the solution is transparent over the whole pH range, even around the isoelectric point of the protein (pH 5.2). This effect is due to the logarithmic correlation between transmission and absorbance. For both peptides a minimum solubility is observed around the isoelectric point of the peptides (pH 8.5, Figure 2). Between pH 5 and 7, the pure protein and the

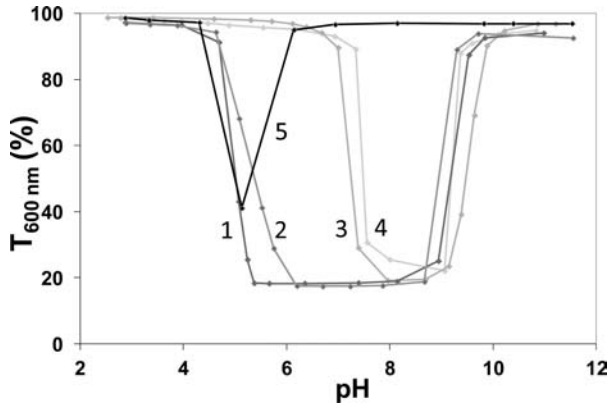

Figure 2. Transmission at 600 nm of (1) β-lactoglobulin + peptide [f135−158], (2) β-lactoglobulin + peptide [f135−162]-SH, (3) peptide [f135−158], (4) peptide [f135−162]-SH, and (5) β-lactoglobulin. Concentration of all components in solution was 5.0 mg/mL in MQ water.

pure peptides are soluble, as indicated by the high transmission at 600 nm. The mixed solutions are not soluble at this pH range, as shown by the decreased transmission in this pH range. This indicates electrostatic association between the peptides and the protein. This agrees with previous results on the binding of peptides and intact β-lactoglobulin at different pH values measured with SELDI-TOF-MS.[9] There it was shown that at low pH (<4.7) the affinity of most peptides to bind to β-lactoglobulin was lower than at higher pH.

The protein−peptide association was further determined by SELDI-TOF-MS for the proteins β-lactoglobulin, ovalbumin, and lysozyme. The peptides did not show nonspecific binding to the SELDI chip under the conditions used in the experiment. The peptides did bind to β-lactoglobulin, ovalbumin, and lysozyme at room temperature (Figure 3). Peptide [f135−158]

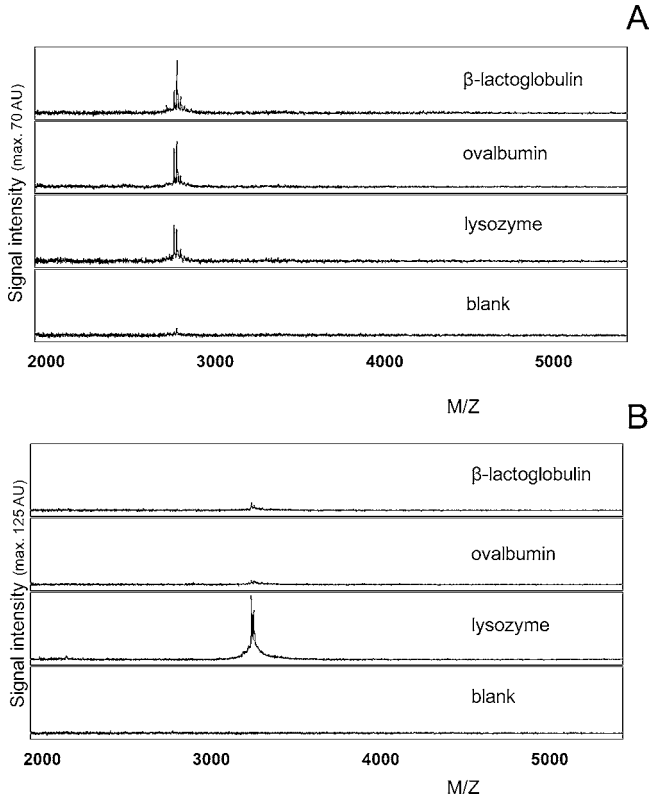

Figure 3. SELDI-TOF-MS spectra of peptide [f135−158] (A) and peptide [f135−162]-SH (B) bound to β-lactoglobulin, ovalbumin, and lysozyme at pH 7.4.

(m/z 2826) bound equally to the three different proteins, as indicated by the similar signal intensity (Figure 3A). In these mass spectra, two masses for this peptide are found. The second mass (m/z 2826 + 16) is due to the oxidation of the methionine residue. The oxidation of methionine is a common modification of methionine-containing peptides.[19] For peptide [f135−162]-SH larger differences were found in the signal intensity after binding to different proteins. The signal of the peptide bound to lysozyme (Figure 3B) was approximately 10 times higher than the signal from the peptides bound to ovalbumin and β-lactoglobulin. The results show that both peptides bind to proteins at room temperature. The binding to β-lactoglobulin was expected on the basis of the decreased solubility at pH 5−7 observed for mixtures of native β-lactoglobulin and peptides at room temperature. The fact that the peptides bind similarly to ovalbumin as to β-lactoglobulin shows that the binding does not depend on specific properties of the protein, such as the retinol binding site of β-lactoglobulin. Rather, both proteins are negatively charged, whereas both peptides are positively charged (+1.0 at pH 7.0).[9] This suggests that the binding is due to electrostatic attraction.

However, [f135−158] binds equally well to lysozyme, whereas the net charge of lysozyme at this pH is also positive (+8.0). This shows that other properties of the peptide such as the total hydrophobicity (31.6 kcal for both peptides)[9] or the influence of the negatively charged residues may be important. It is expected that, on the basis of the similarity of the peptide sequence, both peptides show similar binding behavior. Indeed,

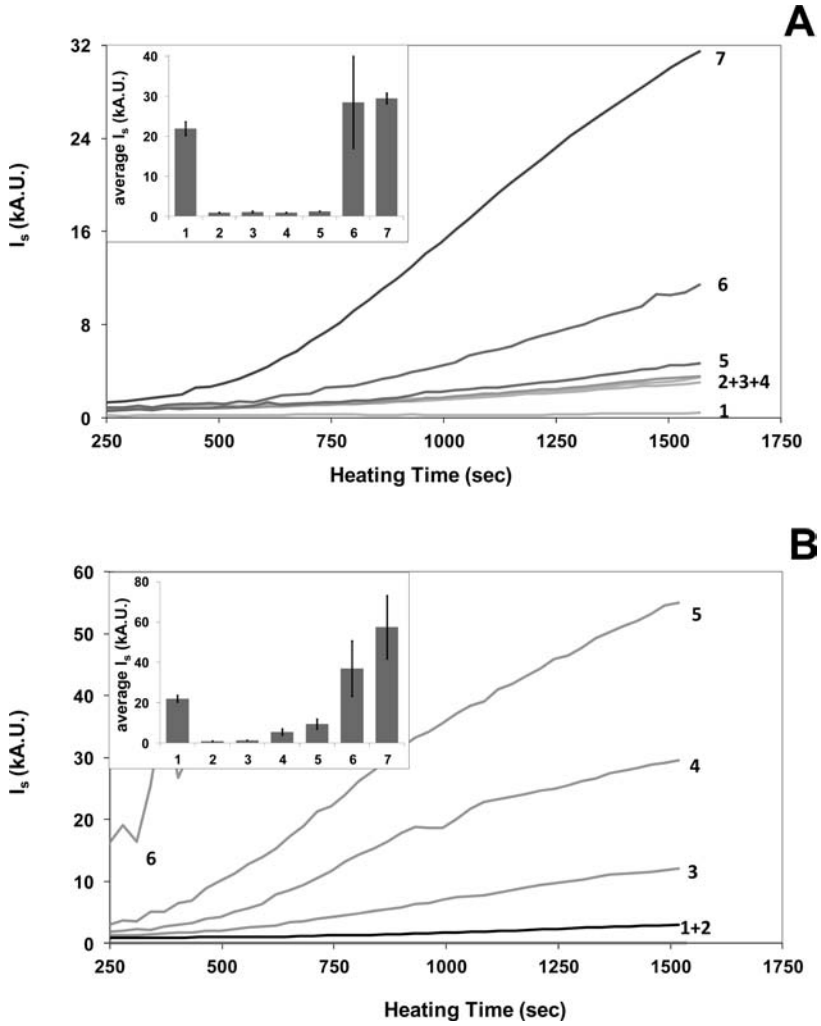

Figure 4. Light scattering intensity of β-lactoglobulin solution during heating at 80 °C from 250 s onward in the presence of peptide [f135−158] (A) and peptide [f135−162]-SH (B). The curves represent (1) only 5.0 mg/mL peptide solution and (2) 5.0 mg/mL β-lactoglobulin and mixtures of 5.0 mg/mL β-lactoglobulin + (3) 0.1 mg/mL, (4) 0.5 mg/mL, (5) 1.0 mg/mL, (6) 2.5 mg/mL, and (7) 5.0 mg/mL peptide solution. All components were dissolved in 50 mM sodium phosphate buffer, pH 7.0. The figures in the inset represent the average light scattering at 20 °C.

the mass intensity of [f135−162]-SH when bound to lysozyme is quite similar to that for [f135−158], but is lower for binding to β-lactoglobulin and even lower for ovalbumin. Still, it was observed that both peptides give a similar association with intact β-lactoglobulin, as determined by turbidity (Figure 2). Therefore, it is concluded that the [f135−162]-SH did bind to β-lactoglobulin in the SELDI-TOF-MS experiment. The lower signal intensity could be due to a lack of desorption during ionization under the conditions used. This could be due to stronger binding or even the formation of a covalent interaction between the free sulfhydryl group of the peptide and a cysteine residue of the protein and, therefore, not detected.

Effect of Binding Peptides on Heat-Induced Aggregation of β-Lactoglobulin. The heat-induced aggregation of β-lactoglobulin in the presence or absence of peptides was studied by DLS experiments (Figure 4 and Table 4). For pure protein the light intensity before and after heating are low (i.e., <5 kAU). Under the conditions used, the unfolding of β-lactoglobulin does not result in the formation of larger aggregates. For pure peptide solutions a high light scattering intensity (LSI) is already observed at 20 °C (inset of Figure 4), indicating peptide−peptide interactions. These peptide−peptide interactions at 20 °C resulting in aggregates were also seen during hydrolysis of WPI by *Bacillus licheniformis* protease.[20] As soon as the peptide solutions were heated to 80 °C, the LSI decreased rapidly (within 250 s) to values below 5

Table 4. Hydrodynamic Diameter (d_h) of β-Lactoglobulin (β-LG) in the Presence of Different Concentrations Peptides [f135−158] or [f135−162]-SH after Heating for 25 min at 80 °C

sample	d_h (nm)
5.0 mg/mL [f135−158]	2
5.0 mg/mL [135−162]-SH	3
5.0 mg/mL β-LG	14
+ 0.1 mg/mL [f135−158]	15
+ 0.5 mg/mL [f135−158]	14
+ 1.0 mg/mL [f135−158]	16
+ 2.0 mg/mL [f135−158]	18
+ 5.0 mg/mL [f135−158]	22
+ 0.1 mg/mL [f135−162]-SH	35
+ 0.5 mg/mL [f135−162]-SH	43
+ 1.0 mg/mL [f135−162]-SH	51
+ 2.0 mg/mL [f135−162]-SH	75
+ 5.0 mg/mL [f135−162]-SH	>400

kAU. This shows that the peptide−peptide interactions or the solubility of the peptides was increased by the higher temperature.

For the β-lactoglobulin−peptide mixtures the LSI at 20 °C increases with the peptide concentration. This is due to protein−peptides complexes that are formed (as also shown in Figure 2). Similarly as observed for the pure peptide solutions, these complexes dissociate rapidly (within 250 s) upon heating at 80 °C.

For [f135−158] at 0.1 and 0.5 mg/mL the LSI stays low, even after longer heating times (up to 30 min). Only at higher concentrations did the LSI increase again at longer heating times, to 32 kAU at 5.0 mg/mL peptide. The peptide concentrations at which heat-induced aggregation starts (1.0 mg/mL) and further increases (2.5 and 5.0 mg/mL) correspond to protein/peptide molar ratios of 1:1.3, 3.3, and 6.5, respectively. This indicates that aggregation of β-lactoglobulin is induced by binding of at least one peptide molecule per protein molecule. A further illustration of the effect of the peptide is seen in the hydrodynamic diameter (d_h) of protein/peptide aggregates. For peptide [f135−158], the aggregate diameter ranges from 14−15 nm for the lowest concentration peptide (0.1 mg/mL) to 22 nm for the highest concentration (5.0 mg/mL) peptide present (Table 4). The same experiments were performed with peptide [f135−162]-SH, which deviates mostly from [f135−158] by the presence of the free sulfhydryl group (Figure 4B). For this peptide, heat-induced aggregation, shown by the LSI (12 kAU), is already observed at the lowest peptide concentration. At the highest peptide concentration also a higher LSI is observed than for [f135−158] (55 and 32 kAU, respectively). The hydrodynamic diameter of heat-induced aggregates are also higher for [135−162]-SH than for [f135−158] and range from 35 nm at 0.1 mg/mL peptide to >400 nm at the highest peptide concentration (5.0 mg/mL). Whereas [f135−158] and [f135−162]-SH both stimulate the heat-induced aggregation of β-lactoglobulin, the peptide with the free sulfhydryl group has a much larger effect. To confirm that the differences are due to the presence of the free sulfhydryl group, it was blocked by NEM. Then, the heat-induced aggregation of β-lactoglobulin in the presence of this peptide, [f135−162]-SX (5.0 mg/mL), was determined. The LSI after heating reached values similar to those obtained with peptide [f135−158], thus confirming the role of the free sulfhydryl group (data not shown). The results show that peptides affect the amount and size of β-lactoglobulin aggregates formed during heating by noncovalent interaction ([f135−158] and [f135−162]-SX), but even more by covalent interactions through sulfhydryl reactivity ([f135−162]-SH). The importance of the free sulfhydryl group was further tested by the addition of equimolar amounts (based on SH in peptide [f135−162]-SH) of the peptide [f159−162]-SH to β-lactoglobulin. The LSI in the presence of peptide [f159−162]-SH was 2 times higher than in the case of β-lactoglobulin with peptide [f135−162]-SH (data not shown). This indicates that the presence of the free sulfhydryl group is already effective in inducing aggregation. In a study on the pressure-induced aggregation of α-lactalbumin it was also observed that more extensive aggregation occurred when cysteine was added to the protein solution.[21]

Peptide-Induced Gelation of β-Lactoglobulin. The complex moduli (G) of heated solutions of β-lactoglobulin in the presence or absence of peptides were determined using the EW technique. For this, first the interfacial tension of the heated droplets needs to be determined. At protein concentrations up to 85 mg/mL the slope of $\Delta\cos(\theta)$ versus U^2 was constant (Figure 5A), indicating that no gelation

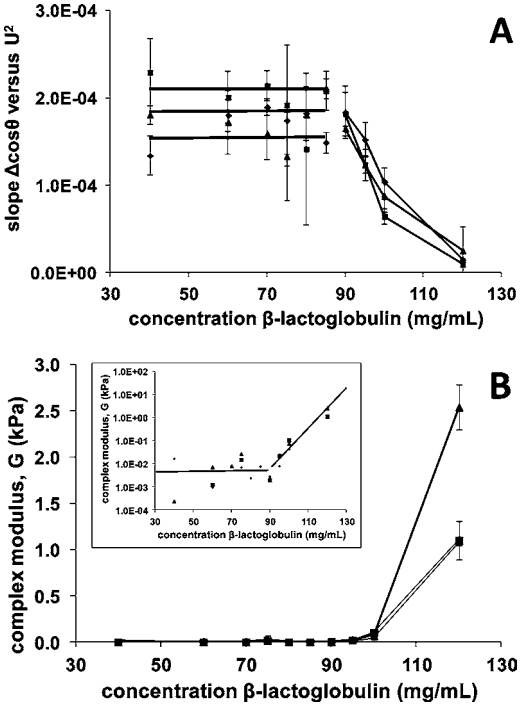

Figure 5. (A) Slope of $\cos(\theta)$ versus U^2 plotted against the concentration of β-lactoglobulin to calculate the interfacial tension (γ) (horizontal lines). (B) Complex modulus of heat-induced gels of β-lactoglobulin (◆) in the presence of peptide [f135−158] (■) or peptide [f135−162]-SH (▲) 50 mM sodium phosphate buffer, pH 7.0. The mass ratio protein/peptide is 48:1. (Inset) Y-axis in log-scale to show minimal gel concentration.

occurred. This agrees with earlier reported values for the minimal gel concentration of β-lactoglobulin under similar conditions.[22] Because in these samples no gelation occurred, the interfacial tension (γ, N/m) for these heated droplets was calculated as the average value obtained at these concentrations. The values were 1.5×10^{-4}, 2.1×10^{-4}, and 1.8×10^{-4} N/m for β-LG, β-LG + peptide [f135−158], and β-LG + peptide [f135−162]-SH, respectively. These values are much lower than those obtained for typical (nonheated) adsorbed protein layers. These lower values are probably due to the heat-induced unfolding of adsorbed proteins. It has been found that heating of adsorbed WPI layers formed at the oil−water interface (from 0.01% protein solutions) resulted in a decrease of surface tension from 15 to 4 mN/m.[23,24] That the values in our experiments are even lower could be due to the higher protein concentrations used. At concentrations >90 mg/mL, the whole droplet gels, resulting in an additional contribution of the complex modulus (G) to $\Delta\cos(\theta)$. For β-lactoglobulin, the calculated G increases to a value of 1.07 ± 0.03 kPa at a concentration of 120 mg/mL (Figure 5B). These values agree with reported values for β-lactoglobulin under the same conditions as determined by traditional rheometry.[25,26]

The presence of peptides does not shift the minimum gel concentration dramatically (≈90 mg/mL, inset Figure 5B). However, for peptide [f135−162]-SH the gel strength at the highest concentration is significantly higher (2.59 ± 0.24 kPa) than that of β-lactoglobulin alone or in the presence of [f135−

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158] (1.10 ± 0.21 kPa). This effect was clearer when the gel strength was compared at a constant concentration (100 mg/mL) of β-lactoglobulin and increasing concentrations of peptides (Figure 6). To correct for the increase in total

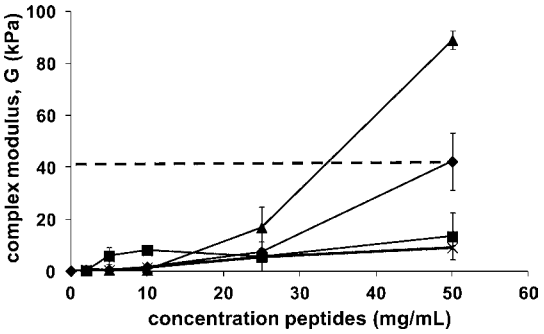

Figure 6. Complex modulus of heat-induced gel of 100 mg/mL β-lactoglobulin in the presence of different concentrations of peptide [f135−158] (■), peptide [f135−162]-SH (▲), peptide [f135−162]-SX (×), or extra β-lactoglobulin (◆) in 50 mM sodium phosphate buffer, pH 7.0. The dashed line indicates the working range (0.01−40 kPa).

proteinaceous material, a reference curve is included where additional β-lactoglobulin was added instead of peptides. At the highest concentration (120 mg/mL + 50 mg/mL β-lactoglobulin) the value was 42 ± 11 kPa. This is in agreement with previous studies on heat-induced β-lactoglobulin gelation under the same conditions.[26] Previously, it was reported that complex moduli obtained with EW experiments were similar to those obtained with Couette rheology for *G* values in the range of 0.03−3 kPa (with gelatin gels).[14] In this work, the complex modulus of pure β-lactoglobulin gels was determined up to a concentration of 50 mg/mL. The complex modulus at that concentration (40 kPa, represented by the dotted line in Figure 6) was found to be similar to the values obtained by Couette rheology.[22] This shows that the working range of the EW technique is valid even for these higher moduli. The complex modulus (*G*) of β-lactoglobulin gel in the presence of the sulfhydryl-containing peptide ([f135−162]-SH) is significantly higher than that for β-lactoglobulin alone and increases to a final value of 89 ± 1 kPa for the highest concentration peptide [f135−162]-SH added (50 mg/mL). For peptide [f135−158], the gel strength at each concentration investigated is lower than that of the reference of β-lactoglobulin alone. This indicates that at constant concentration of proteinaceous material the presence of this peptide effectively decreases the gel strength, whereas at constant β-lactoglobulin concentration the presence of this peptide has no significant effect. Still, the aggregation behavior was found to be affected. The role of reactive sulfhydryl groups from the protein on the gel strength has been described.[27,28] It has been proposed that the sulfhydryl groups on intact proteins can attack existing disulfide bridges in other proteins, initiating a thiol/disulfide exchange reaction leading to different unfolded states and covalent linkages between different types of protein aggregates. The results in this paper show that the addition of free sulfhydryl in the form of peptides also increases the gel strength. It must be considered that the peptides contain only one sulfhydryl group and in that way they can take part in only one link. However, the attack of the sulfhydryl group on existing disulfide bridges releases free sulfhydryl groups on the protein, which in turn may increase the gel strength. It may be that the macroscopic gel properties are affected but that these changes are not reflected in the *G* as measured in these experiments. Peptide [f135−162]-SX, in which the sulfhydryl group is blocked, shows a behavior similar to that of peptide [f135−158]. The gel strength is lower than for β-lactoglobulin alone (9.2 ± 0.7 and 42 ± 11 kPa, respectively) and is almost equal to that for peptide [f135−158] (14 ± 1 kPa). The increase in gel strength after heat-induced gelation of β-lactoglobulin due to the availability of additional sulfhydryl groups has also been demonstrated by others.[29,30] These results clearly show the importance of the free sulfhydryl group in the gelation mechanism and gel strength of β-lactoglobulin. Moreover, the gel strength of β-lactoglobulin can be directed toward both lower and higher values.

In conclusion, the binding of peptides to the protein results in increased amounts of heat-induced aggregates of the protein. In a concentrated system these differences in aggregation behavior lead to other gel properties and can be directed by the type of peptide. The sulfhydryl group in the peptide plays an important role in the amount of protein aggregates formed and increases the gel strength of a concentrated protein system.

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Notes

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