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Physical and Chemical Interactions in Cold Gelation of Food Proteins

ARNO C. ALTING,*,†,‡ HARMEN H. J. DE JONGH,†,§ RONALD W. VISSCHERS,†,‡ AND JAN-WILLEM F. A. SIMONS†,||

Wageningen Centre for Food Sciences, Diedenweg 20, 6700 AN Wageningen, The Netherlands; NIZO Food Research, P.O. Box 20, 6710 BA, Ede, The Netherlands; Wageningen University and Research Centre, Wageningen, The Netherlands; and TNO Nutrition and Food Research, Zeist, The Netherlands

pH-Induced cold gelation of whey proteins is a two-step process. After protein aggregates have been prepared by heat treatment, gelation is established at ambient temperature by gradually lowering the pH. To demonstrate the importance of electrostatic interactions between aggregates during this latter process, β-lactoglobulin aggregates with a decreased iso-electric point were prepared via succinylation of primary amino groups. The kinetics of pH-induced gelation was affected significantly, with the pH gelation curves shifting to lower pH after succinylation. With increasing modification, the pH of gelation decreased to about 2.5. In contrast, unmodified aggregates gel around pH 5. Increasing the iso-electric point of β-lactoglobulin via methylation of carboxylic acid groups resulted in gelation at more alkaline pH values. Comparable results were obtained with whey protein isolate. At low pH disulfide cross-links between modified aggregates were not formed after gelation and the gels displayed both syneresis and spontaneous gel fracture, in this way resembling the morphology of previously characterized thiol-blocked whey protein isolate gels (Alting, et al., *J. Agric. Food Chem.* **2000**, *48,* 5001−5007). Our results clearly demonstrate the importance of the net electric charge of the aggregates during pH-induced gelation. In addition, the absence of disulfide bond formation between aggregates during low-pH gelation was demonstrated with the modified aggregates.

KEYWORDS: β-lactoglobulin; whey protein isolate; chemical modification; aggregation/gelation; electrostatic interactions; disulfide bonds

INTRODUCTION

Food protein gels are often formed during heating, and consequently these are referred to as heat-induced or heat-set gels (*1*, *2*). For some proteins, however, a gelation method at ambient temperatures has been reported (*3*). This so-called cold gelation consists of two consecutive steps. In the first step, aggregates are formed by heating a protein solution for a certain period of time. Upon subsequent cooling, the protein aggregates remain soluble and can be stored for days without occurrence of significant changes in aggregate size or other properties (*4*). In the second step gelation is induced by changing the solvent quality, for example by the addition of calcium or sodium or by lowering the pH. A typical acid-induced cold-set gel is formed by a gradual and slow acidification of the solution of protein-aggregates by addition of glucono-δ-lactone (GDL). In aqueous solutions this component slowly hydrolyzes to gluconic acid, causing a gradual lowering of the pH (*5*).

* To whom correspondence should be addressed. Phone: +31 318 659571. Fax: +31 318 650400. E-mail: Alting@NIZO.nl.
† Wageningen Centre for Food Sciences.
‡ NIZO Food Research.
§ Wageningen University and Research Centre.
|| TNO Nutrition and Food Research.

Because cold gelation provides the possibility to introduce gel structures into foodstuffs without the need to heat the final product, it provides an attractive alternative for currently used thickening ingredients (mostly carbohydrates) (*3*). Cold gelation of heat-treated solutions has been reported for purified β-lactoglobulin (β-Lg), crude whey protein concentrates, and whey protein isolates (*6*−*11*).

Compared to the wealth of information that is available on the mechanism and kinetics of heat-set aggregation and gelation (i.e., *2*, *12*−*14*), relatively few papers have been published that deal with specific properties of ingredients or the importance of preparation conditions for cold gelation (*7*, *10*, *11*, *15*, *16*). As a result, there are still a number of unanswered questions regarding the cold gelation process. In particular, the relative contribution and importance of physical (electrostatic and hydrophobic) versus chemical (disulfide bond formation) interactions in the aggregation and gelation process is still not understood at the molecular level. The role of electrostatic interactions has been demonstrated in the past by the addition of salts to shield the electric charge of the proteins and by studying the pH-dependency of gelation (*17*, *18*). These approaches do not only result in a change of the net charge of

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the protein, but also have potential side-effects such as a promotion of hydrophobic interactions. Therefore, these experiments do not provide direct proof regarding the importance of electrostatic interactions in gelation. With respect to the formation of intermolecular disulfide bonds, Alting et al. (*4*) have recently demonstrated the importance of these interactions for the mechanical properties of cold-set, acid-induced gels of whey protein isolate (WPI). The formation of disulfide bonds predominantly occurs under alkaline conditions (*4*). Alting et al. (*4*) have shown that formation of disulfide bonds also occurs in acid-induced cold-set gels at pH 5. We postulated that the formation of disulfide bonds under these conditions was attributed to a large increase of the effective protein concentration and therefore of the effective concentrations of thiol groups. It was shown that formation of disulfide bonds increased the molecular weight of the aggregates formed during gelation, and these bonds were involved in stabilizing the network, resulting in a much stronger gel (*4*). However, the common notion is that noncovalent interactions play a dominant role in the initial formation of gels and aggregates.

In this paper, we used a combination of chemical modification of aggregates and acid-induced gelation to study and control the balance between the role of electrostatic and chemical interactions in acid-induced cold gelation. Pure β-Lg aggregates were applied as a model system, of which calculation of the iso-electric point after modification is possible. Because most studies on the process of cold gelation were performed with WPI, aggregates of this mixture of proteins were also involved in this study.

MATERIALS AND METHODS

Materials. Bovine milk β-Lg was purified from fresh milk using a nondenaturating protocol as described recently (*19*). The whey protein isolate (WPI) Bipro was obtained from Davisco International Inc. (La Sueur, MN). The WPI consisted (based on dry weight) of β-Lg (74%), α-lactalbumin (12.5%), bovine serum albumin (5.5%), and immunoglobulins (5.5%). The total amount of proteins in the powder was 97.5%, and it further contained lactose (0.5%) and ash (2%) (*20*). Succinic anhydride was purchased from Fluka. Ortho-phthaldialdehyde (OPA) and glucono-δ-lactone (GDL) were bought from Sigma. *N*,*N*-dimethyl-2-mercaptoethylammonium chloride (DMA) and di-sodium tetraborate decahydrate (Borax buffer) were purchased from Merck. Sodium dodecyl sulfate (SDS) and Triton X-100 were from Serva. Electrophoresis grade agarose was obtained from Life Technologies (Paisley, Scotland). Phast blue R tablets were from Pharmacia Biotech (Uppsala, Sweden).

Preparation of Aggregates. β-Lg was dissolved in double-distilled water at ambient temperature at a protein concentration of 9% (w/w). The pH of the solution was adjusted to 7.2. Reactive β-Lg aggregates (9% w/w) were prepared by heating the β-Lg-solution in a water bath for 4 h at 68.5 °C.

WPI was dissolved in double-distilled water at a protein concentration of 9% (w/w) and stirred for at least 2 h at ambient temperature (*21*). The pH after solubilization was 7.2. Reactive WPI aggregates were prepared by heating the WPI solution in a water bath for 2 h at 68.5 °C (*20*). After heating, both the solution of β-Lg aggregates and the solution of WPI aggregates were rapidly cooled to 20 °C by using running tap water. The solutions of aggregates were diluted with double-distilled water to 4.5% (w/w) before modification and stored at 4 °C until use (typically within 1 week).

Succinylation. Amino groups in a 4.5% solution of β-Lg aggregates (2.5 mM; 16 amino groups) or WPI aggregates were succinylated essentially as described by Klotz (*22*). To this end, the solutions of aggregates were adjusted to pH 8 by the addition of 1 M NaOH. To aliquots of 40 mL of solution of β-Lg aggregates, respectively 47, 94, 141, and 280 mg of solid succinic anhydride was stepwise added in 10 portions, yielding final concentrations of 12 (further denoted as sample β-Lg a), 24 (β-Lg b), 35 (β-Lg c), and 70 (β-Lg d) mM succinic anhydride. To aliquots of 65 mL of solution of WPI aggregates, respectively 150 and 750 mg of solid succinic anhydride were stepwise added in 10 portions yielding final concentrations of 23 (WPI a) and 115 (WPI b) mM succinic anhydride. During the addition of succinic anhydride, the pH was kept at 8 ($\pm$ 0.2) by the addition of 1 M NaOH using a pH-stat. After the treatment with succinic anhydride, the excess of reagents was removed by extensive dialysis (3× an 80-fold excess) against deionized water at 4 °C. Nontreated aggregates incubated at pH 8 were also dialyzed and used as a reference material. After dialysis, the sample volume was significantly increased. The solutions of aggregates were further diluted to a final protein concentration of 1.5% (w/w). Sodium azide (0.02% final concentration) was used as a preservative.

Chromogenic OPA Assay to Determine the Degree of Succinylation. The degree of succinylation was determined by the method described by Schmidt and Van Markwijk (*23*). This method is based on the specific reaction between ortho-phthaldialdehyde (OPA) and free primary amino groups in proteins in the presence of DMA, resulting in alkyl-iso-indole derivatives that show an absorbency at 340 nm. The OPA reagent was freshly prepared by dissolving 40 mg of OPA in 1 mL of methanol, followed by the addition of 25 mL of 0.1 M Borax buffer, 200 mg of DMA, and 5 mL of 10% SDS. Finally, the volume was adjusted to 50 mL with deionized water. A quartz cuvette was filled with 3 mL of this reagent and the absorbency at 340 nm was determined. Subsequently, 30 μL of a β-Lg or WPI aggregates solution was added, and after an incubation time of 20 min at room temperature, the absorbance at 340 nm was determined again. A calibration curve was obtained by adding 10, 20, 30, 40, 80, 100, and 150 μL of a 2 mM L-leucine solution in water to 3 mL of OPA reagent, yielding concentrations in the range from 6.6 to 95 μM l-leucine. All measurements were performed in triplicate.

Chromogenic Ellman's Assay to Determine the Number of Free Thiol Groups. The amount of accessible thiol groups at the surface of the aggregates before and after modification was determined using 2-nitro-5-mercaptobenzoic acid (DTNB), also known as Ellman's reagent (*24*). The assay was performed in the absence of urea and SDS, because only the thiol groups on the surface of the aggregates had to be determined. The number of thiol groups was calculated using a molar extinction coefficient for DTNB of 13 600 M^{-1} cm^{-1}.

Fluorescence Quenching Experiments. Fluorescence quenching experiments were carried out with solutions of 0.015% (modified) β-Lg aggregates ($A_{280\ nm}$ < 0.2) in 20 mM Tris−HCl buffer at pH 7. The fluorescence intensity was measured at the maximum intensity at a wavelength of 338 nm, using excitation at 280 nm, in a luminescence spectrometer (Perkin-Elmer, LC50B). Small aliquots of 5 M of the quencher, acrylamide, were mixed with solutions of (modified) β-Lg. Final concentrations ranged from 0 to 100 mM acrylamide. The relative fluorescence was plotted versus the quencher concentration.

GDL-Induced Aggregation. GDL was added as a powder to the β-Lg and WPI solutions (concentration of protein 1.5%) to induce cold gelation at ambient temperature. Because proteins are buffering components, the total amount of GDL added to reach a certain pH value depends on the protein concentration (*5*). To observe gelation of the nontreated aggregates at different pH values, increasing amounts of GDL were added. To solutions of aggregates (5 mL) in glass tubes, 0, 0.075, 0.15, 0.20, 0.25, 0.5, 1, 2, 4, or 6% (w/w) GDL was added. The pH was determined after 24 h of incubation at ambient temperature. Gelation was observed by visual inspection.

Turbidity Measurements. Turbidity measurements were performed at 20 °C and at a protein concentration of 1.5% on a Cary 1E UV−Vis spectrophotometer (Varian) equipped with a temperature controller. The turbidity was measured in time as the absorbency at 500 nm. Samples were measured in cuvettes with a path length of 2 mm. The pH was monitored simultaneously in samples placed in a water bath kept at 20 °C. Such an amount of GDL was added that the dependence of the turbidity could be monitored over the pH range from 7 to 2.5. Because of a difference in buffering capacity of the modified aggregates, different amounts of GDL had to be added to solutions of nontreated and treated aggregates, 1% or 6%, respectively.

Table 1. Effect of Cold Gelation on the Hydrodynamic Diameter of β-Lg and WPI Aggregates for Nonmodified and Succinylated Aggregates

sample	hydrodynamic diameter (nm)[b]	
	before gelation	after gelation
β-lg (nonmodified)	80	172
β-lg a[a]	83	111
β-lg b[a]	89	75
β-lg c[a]	114	68
β-lg d[a]	145	75
WPI (nonmodified)	75	>1000
WPI a[a]	81	208
WPI b[a]	176	118

[a] Aggregates treated with different amounts of succinic anhydride (see Materials and Methods). [b] The index of polydispersity varied between 0.3 and 0.4.

Characterization of Aggregate Size. SDS-agarose continuous gel electrophoresis (0.4% (w/w) agarose) was performed to determine the differences in molecular weight of the different treated β-Lg and WPI aggregates. The electrophoresis buffer consisted of 100 mM Tris−HCl, 50 mM sodium acetate, 2 mM EDTA, and 0.1% SDS, and was adjusted to pH 7.9 with glacial acetic acid. Aggregates (in solution or gel) were mixed with 2 parts of 20 mM Bis−Tris buffer (pH 7.0) containing 5% SDS and were held at ambient temperature while constantly being stirred (final protein concentration was 0.5%). After overnight incubation no gel particles could be observed with a standard microscope (at 400× magnification). Prior to electrophoresis, 5% of a solution containing 60% glycerol and 0.002% bromophenol blue was added. The gels were run with a constant voltage of 50V for approximately 2 h. Staining was done with Phastgel Blue R (Pharmacia Biotech, Uppsala, Sweden). An alternative method was used to improve staining of the more negatively charged aggregates (*25*). In short, 1 tablet of Phastgel Blue R was dissolved in a solution of 15 g $Al(NO)_3$ in 256 mL of deionized water. To this solution 100 mL of 2-propanol (25%), 40 mL of acetic acid (10%), and 4 mL of Triton X-100 were added. This solution was filtered before use. Destaining of the gels was done with 7% acetic acid in demineralized water.

Dynamic light scattering (DLS) experiments were performed as outlined by Verheul et al. (*14*). Experiments were performed using a Malvern Autosizer IIC submicron particle size distribution analyzer. The system consisted of a Malvern PCS41 optics unit with a 5 mW He−Ne laser, and a Malvern K7032-ES correlator used in serial configuration. The Autosizer IIC worked at a fixed scattering angle of 90°, and the wavelength of the laser beam was 632.8 nm. Both the scattering intensity and the apparent diameter were evaluated. The quartz cuvette (10 mm) containing the sample was thermostated by a Joule−Peltier thermostat (20 °C). The apparent diameter of the aggregates in solution was calculated from a cumulant fit of the intensity autocorrelation function. Before analysis, samples were filtered through a low-protein binding membrane (5 μm; Millex-SV, Millipore Corporation, Bedford, MA).

Figure 1. Agarose gel electrophoresis (in the absence of SDS) of nonmodified and succinylated WPI aggregates. Proteins were stained according to the standard Coomassie brilliant blue staining method: lane 1, nonmodified WPI-aggregates; lane 2, 56% succinylated WPI-aggregates; lane 3, 93% succinylated WPI aggregates.

Table 2. Characterization of β-Lg and WPI Aggregates

sample	concentration of amino groups		degree of modification (%)	calculated pI-shift[c]	shift in pH/turbidity curve[d]	concentration of thiol groups (mM)[e]
	mM[b]	mol/mol protein				
β-lg (nontreated)	12.4	15.2	0	-	-	0.16
β-lg a[a]	8.7	10.7	30	−0.4	−0.4	0.16
β-lg b[a]	3.9	4.8	69	−0.9	−0.7	0.22
β-lg c[a]	2.1	2.6	83	−1.1	−0.9	0.26
β-lg d[a]	0.5	0.6	96	−1.3	−1	0.29
WPI (nontreated)	12.2	n.d.	0	n.d.	-	0.17
WPI a[a]	5.4	n.d.	56	n.d.	−0.8	0.23
WPI b[a]	0.9	n.d.	93	n.d.	−1.3	0.29

[a] Aggregates samples treated with different amounts of succinic anhydride (see Materials and Methods). [b] The number of free primary amino groups is determined according to Schmidt and van Markwijk (*23*). This method is based on the specific reaction between ortho-phthaldialdehyde (OPA) and free primary amino groups in proteins. Measurements were done in duplicate with an experimental error lower than 10%. [c] Theoretical shift of pI on the basis of changes in the protein titration curve upon conversion of positive into carboxyl groups. [d] Determined shift in pH/turbidity curve determined as the shift in pH at an absorbance of 1 (**Figure 3**). [e] Determined at pH 7 with Ellman's assay (*24*). The number of thiol groups was determined using ϵ(412 nm) = 13 600 M^{-1} cm^{-1} for 2-nitro-5-mercaptobenzoic acid and expressed as the concentration thiol groups (mM) in a 1.5% (w/w) dispersion of protein aggregates. Measurements were done in duplicate with an experimental error lower than 10%. n.d., not determined, because WPI is a mixture of proteins.

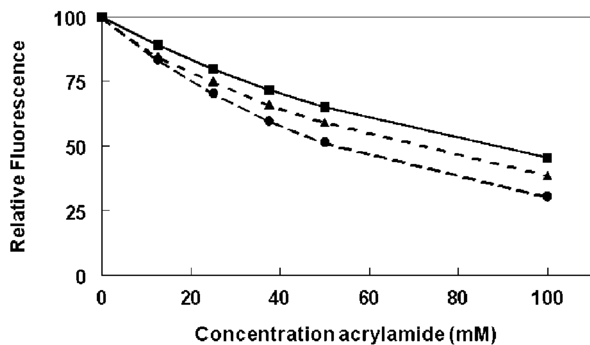

Figure 2. Relative fluorescence of solutions of β-Lg aggregates as a function of the concentration acrylamide. Experimental conditions: 0.15 mg/mL β-Lg; 20 mM Tris (pH 7); $\lambda_{ex} = 280$ nm; $\lambda_{em} = 338$ nm. The experimental error was less than 10%. Squares, nonmodified aggregates; triangles, 69% succinylated aggregates; circles, 96% succinylated aggregates.

RESULTS AND DISCUSSION

Preparation of Aggregates. As a first step in the process of cold gelation, protein aggregates of β-Lg and WPI were prepared. Heat treatment of both a β-Lg and a WPI solution (9%) at neutral pH resulted in a dispersion of soluble aggregates with hydrodynamic diameters of approximately 80 nm, as determined by dynamic light scattering (**Table 1**), well within the range of previously reported sizes (*4*, *11*, *26*). SDS-agarose electrophoresis (see below) confirmed the equality in size. After cooling to room temperature, prior to modification, the dispersion of aggregates was diluted to 4.5% (w/w). After modification, the dispersion was diluted to 1.5% (w/w) and stored at 4 °C. During storage for up to 7 days no significant changes in the size of the formed aggregates were observed as determined with electrophoresis and light scattering techniques.

Modification of Aggregates. To study the possibility of preparing cold-set gels in more acidic regions, the net charge of the aggregates was modified by reaction of the primary amino groups (lysine- residues) present on the aggregates with the reagent succinic anhydride. As this modification results in an inversion of the positively charged amino groups into negatively charged carboxylic acid groups, it has a large effect on the net charge of the protein aggregates. A range of β-Lg and WPI preparations with increasing modification degrees was made by adding different amounts of succinic anhydride during the modification. The degree of modification was determined by titration of the free amino groups with the chromogenic reagent ortho-phthaldialdehyde. The data in **Table 2** show that a set of aggregates for both β-Lg and WPI was prepared with clearly different degrees of modification. Note that for the nonmodified β-Lg aggregates, within the experimental error, the theoretical number of sixteen amino groups per mole of protein was determined, and that this number decreased upon modification.

Aggregate Characterization. In this study we focused on the role of the net electric charge of the aggregates in pH-induced gelation. Therefore, we used succinic anhydride, which is expected to change the net charge of the aggregates by inversing the charge of reacting amino groups. To demonstrate the change in the net charge of the succinylated aggregates, we used a modification of the previously described method of agarose gel electrophoresis (*4*). The gel was run in the absence of SDS to separate the aggregates on the basis of their net electric charge rather than on the basis of their size alone. As expected from their increased negative charge, the succinylated aggregates migrated more toward the anode than the nonmodified aggregates (**Figure 1**). To check if succinylation also led to chemical cross-linking reactions between aggregates, we used the agarose electrophoresis technique in the presence of SDS. This technique was shown to be suitable to separate protein (aggregates) with a diameter ranging from 3 (monomeric protein) to approximately 250 nm. The gels shown in **Figure 3** clearly illustrate that aggregates and monomeric protein can be separated with this technique, because the electrophoretic

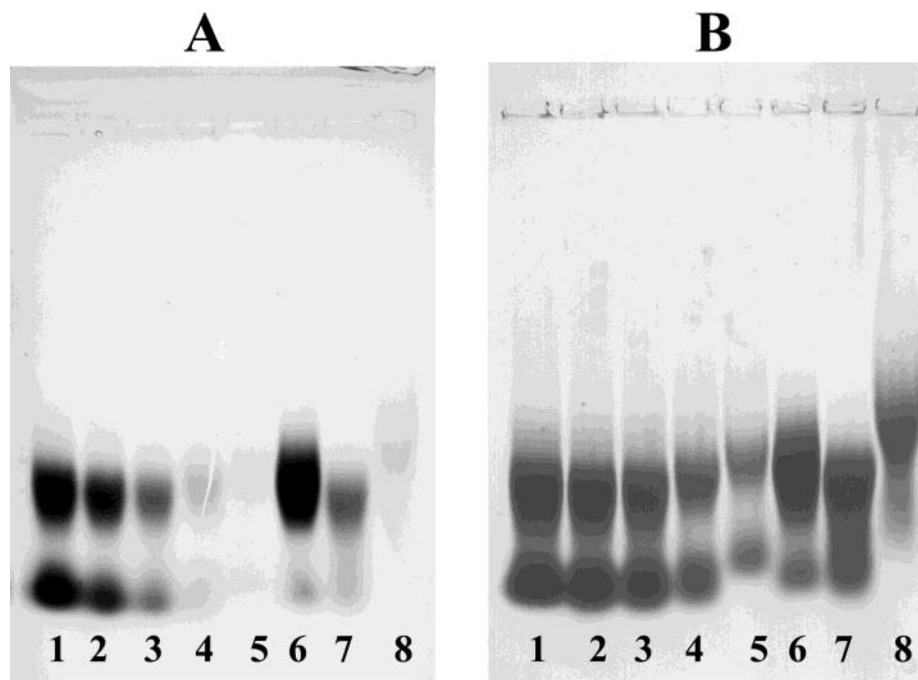

Figure 3. SDS-agarose gel electrophoresis of nonmodified and succinylated β-Lg and WPI aggregates. Proteins were stained according to the standard Coomassie brilliant blue staining method (gel A) and according to an adapted method from Hegenauer et al. (*25*) (gel B). β-Lg aggregates: lane 1, nonmodified; lane 2, 30% succinylated; lane 3, 69% succinylated; lane 4, 83% succinylated; lane 5, 96% succinylated. WPI aggregates: lane 6, nonmodified; lane 7, 56% succinylated; lane 8, 93% succinylated.

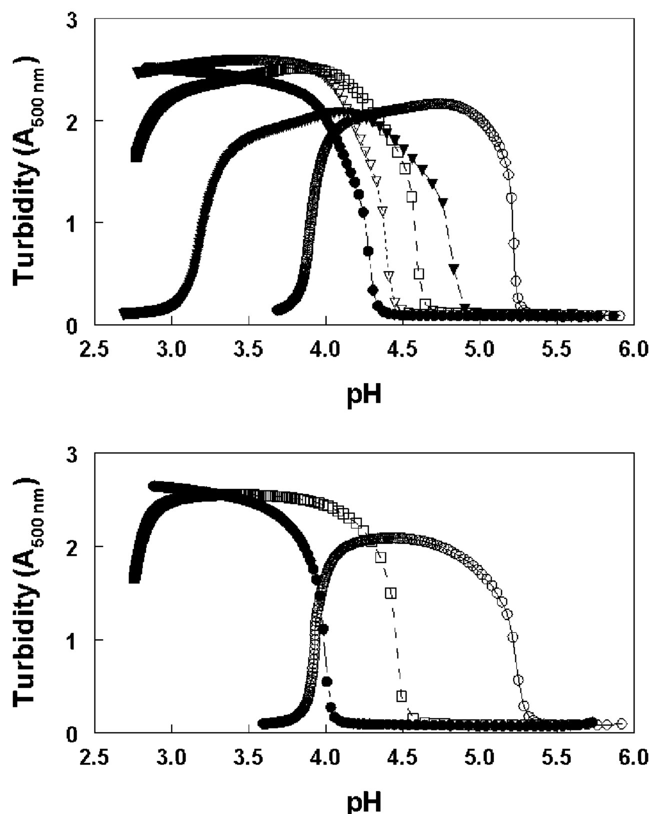

Figure 4. Dependence of the turbidity on the pH for solutions of nonmodified and succinylated β-Lg (A) and WPI-aggregates (B). The turbidity was measured in time as the absorbance at 500 nm. Samples were measured in cuvettes with a path length of 2 mm. The pH was monitored simultaneously. Turbidity measurements were performed at 20 °C. The experimental error was less than 5%. Note that at higher pH values fewer data points were collected than at lower pH values, because GDL-induced acidification is not linear, while data collection took place at constant time intervals. A β-Lg aggregates: open circles, nonmodified; closed triangles, 30% succinylated; open squares, 69% succinylated; open triangles, 83% succinylated; closed circles, 96% succinylated. B WPI aggregates: open circles, nonmodified; open squares, 56% succinylated; closed symbol, 93% succinylated.

mobility of the aggregates (upper bands) is decreased compared to the mobility of the monomeric protein (lower bands). Although for both the β-Lg and the WPI solutions, the majority of the protein is present as aggregates, the amount of residual monomeric protein after heat treatment for β-Lg is higher than that for WPI. The electrophoretic mobility of the moderately modified (up to 50%) β-Lg and WPI aggregates does not differ from the nonmodified aggregates, but the highly modified aggregates show a slight, but reproducible, decrease in electrophoretic mobility suggesting a small increase in aggregate size or swelling of the aggregates. Note that this effect is larger for the WPI aggregates as compared to the β-Lg aggregates. The results of the electrophoresis experiments are confirmed by the determination of the hydrodynamic diameter of the nonmodified aggregates (before gelation) by the use of light scattering (**Table 1**).

It was observed that the more intensively succinylated aggregates were difficult to stain using the standard Coomassie staining method (**Figure 3A**). Therefore, an adapted method was used (**Figure 3B**), which was developed to improve staining of highly acidic proteins (*26*). Staining of the most intensively succinylated aggregates improved with this method (compare lanes 5 and 8 in **Figure 3A** to lanes 5 and 8 in **3B**). The results show again that the succinylated aggregates are more negatively charged than the nonmodified aggregates, while the aggregate size has remained comparable to that of the nonmodified aggregates, except for the most intensively succinylated species.

The number of accessible thiol groups after modification was detected, because it is known that the thiol groups play an important role during acid-induced gelation (*4*). The concentrations of thiol groups of a 1.5% (w/w) dispersion of the nonmodified β-Lg and of WPI aggregates are the same within experimental error (**Table 2**). Surprisingly, the concentration of accessible thiol groups increased upon increasing the modification degree, both for the β-Lg and the WPI aggregates. Because the used succinic anhydride is highly specific for free amino groups, no reaction with thiol groups, and therefore no direct effect on the number of reactive thiol groups, was expected. However, as a result of the succinylation reaction the net negative charge of the aggregates will have increased. As a consequence the voluminosity of modified aggregates could have increased, either by swelling caused by the increase of electrostatic repulsion within the aggregates or by an increase of the excluded volume due to the poly-electrolyte effect. Indeed, both an increase in the hydrodynamic diameter (**Table 1**) and a decrease in the electrophoretic mobility (**Figure 3**) were observed with increasing degree of succinylation. The increase in voluminosity does not directly explain the observed increase in the amount of thiol groups accessible for the Ellman's reagent. To verify this, tryptophan fluorescence quenching was used to demonstrate that small molecules can penetrate more easily into the swollen (modified) aggregates. From the results shown in **Figure 2** it is clear that the tryptophan fluorescence of the nonmodified β-Lg aggregates decreases upon addition of acrylamide. For the succinylated aggregates, this decrease is more pronounced and depends on the degree of modification. This demonstrates that the quencher (acrylamide) can more effectively reach the intrinsic tryptophans in the modified aggregates. In a similar fashion, the Ellman's reagent can probably react more efficiently with the thiol groups within the swollen aggregates, due to their increased accessibility for small chemical compounds.

pH-Induced Gelation. To test whether the differences in net electric charge result in different pH-induced gelation behavior, we followed the turbidity of aggregate dispersions as a function of the pH. To this end, a relatively large amount of solid GDL was added to gradually decrease the pH of the protein aggregate solution (from pH 7 to approximately pH 2.5). At a pH near the iso-electric point of nonmodified β-Lg (5.1) the electrostatic repulsion between the negatively charged aggregates is reduced and therefore aggregation through physical interactions is promoted. As a result, the turbidity increases (**Figure 4A**, open circles) and a turbid gel is being formed (see below). The pH continues to decrease in the gel, and below pH 4.5 (well below the iso-electric point of β-lactoglobulin) the turbidity starts to decrease. We attribute this phenomenon to the increased electrostatic repulsion, in this case of positively charged aggregates. As the acidification rate is relatively high in these experiments, extensive formation of disulfide cross-links between aggregates (time scale of hours) does not occur during the gel state (*4*). The absence of disulfide cross-links enables the gel to dissolve at pH values below the iso-electric point. Therefore, the data displayed in **Figure 4** could be interpreted as a sol−gel−sol transition as the pH decreases from 7 to 2.5. A similar aggregation curve was obtained for the WPI aggregates (**Figure 4B**). It is clear from **Figure 4** that the aggregation curves for the modified aggregates have shifted

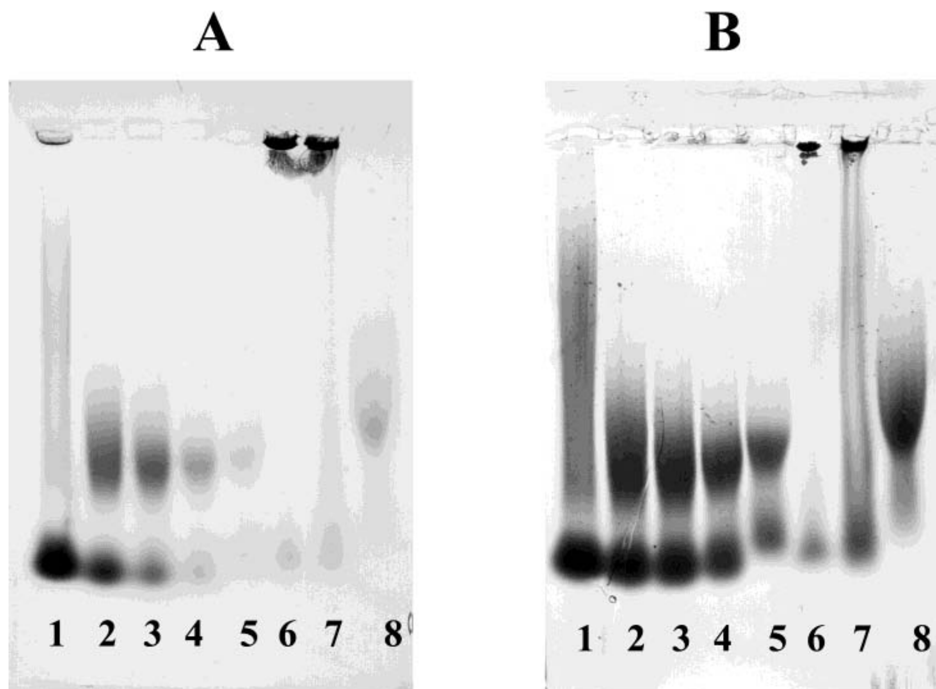

Figure 5. SDS-agarose gel electrophoresis of nonmodified and succinylated β-Lg and WPI aggregates after gelation and resolubilization in a SDS-containing buffer at pH 7. Proteins were stained according to the standard Coomassie brilliant blue staining method (gel A) and according to an adapted method from Hegenauer et al. (*25*) (gel B). β-Lg aggregates: lane 1, nonmodified; lane 2, 30% succinylated; lane 3, 69% succinylated; lane 4, 83% succinylated; lane 5, 96% succinylated. WPI aggregates: lane 6, nonmodified; lane 7, 56% succinylated; lane 8, 93% succinylated.

significantly to the more acidic region, both for the β-Lg and the WPI aggregates. The observed shifts correspond well with the earlier determined degree of modification (**Table 1**). Moreover, the calculated shift in iso-electric points of the succinylated aggregates is in good agreement with the shift in pH at the inflection point in **Figure 4A** (**Table 1**). For the highest degree of modification, the onset point of aggregation was shifted more than one pH unit toward lower pH. As far as we know, this is the first time that the importance of the net electric charge was demonstrated by intrinsic modifications at the protein level and not by changing the environmental conditions.

Apart from the shift toward lower pH, there are remarkable differences in the kinetics of the aggregation, more specifically in the maximums of the turbidity curves. We speculate that these differences might be related to changes in structural properties of the protein network caused by rearrangements, which are expected to be influenced by the formation of additional disulfide bonds. As these differences are found in an area close to the limit of linearity of the spectrophotometer, care should be taken with the interpretation of these data.

To characterize in more detail the influence of pH on the properties of the gels formed from the different treated aggregates, additional experiments were performed. In the previous described aggregation experiments an excess of GDL was added to create a continuous pH gradient in time. In contrast, in this experiment fixed amounts of GDL were added to set each system at a predetermined pH at equilibrium to investigate the gel properties independently of time. The results showed that with the most extensively modified aggregates (>70%) gels could be formed even at pH 2.5, while the nonmodified aggregates gelled in the pH region 4−5.5 (not shown). Another important observation was the occurrence of syneresis and spontaneous rupture in the gels made from more intensively modified aggregates (after 24 h of incubation at ambient temperature) formed in the low pH region (below pH 3). It should be noted that this effect was most clear for the gels made from modified WPI aggregates (not shown). The appearance of these gels formed at pH < 3 was comparable to that of gels earlier prepared from differently modified WPI (*4*). In this case the WPI aggregates were modified with a thiol blocking reagent, preventing the formation of disulfide bonds during gelation at pH 5. This raised the question whether the microstructure of gels formed in the pH region below 3 is stabilized by formation of new disulfide bonds.

Degree of Disulfide Bond Formation. To study the formation of covalent disulfide bonds, gels were dissolved in a buffer containing SDS to break physical interactions while the covalent bonds remained intact. Gels formed in the low pH region dissolved quite easily. This was also observed for the thiol-blocked gels as reported earlier (*4*). We used agarose electrophoresis to characterize the size of the aggregates formed after solubilization of the gels. Gel samples taken at the maximum of the pH−turbidity curves (**Figure 4**) were dissolved in a Tris buffer (pH 7) containing SDS. Clear differences in electrophoretic mobility were observed between these aggregates (**Figure 5**). Both for the nonmodified β-Lg and WPI aggregates, the electrophoretic mobility has significantly decreased after gelation, suggesting the formation of large covalently cross-linked aggregates (diameter > 250 nm). It has already been shown for WPI aggregates that such larger aggregates are linked via disulfide bonds, since they easily dissociate after the addition of dithiothreitol (*4*). For the modified aggregates (both β-Lg and WPI) this increase in aggregate size was less pronounced. For the highest degree of modification, the aggregate size after gelation was identical to that before gelation, illustrating the complete absence of newly formed disulfide cross-links in the gels at pH 2.5. Because even more accessible thiol groups could be determined with increasing degree of modification, under these conditions, formation of disulfide bonds is clearly not possible.

The size of the aggregates before and after gelation was also determined by dynamic light scattering. From **Table 1** it is clear that the size of the aggregates before gelation increases upon modification, most significantly for the highest degree of modification. This is in agreement with the results from agarose electrophoresis experiments (**Figure 3**), and with the results from fluorescence quenching experiments (**Figure 2**). With respect to the aggregate size after gelation, the results show that a clear increase in hydrodynamic diameter occurred only with the nonmodified and less intensively modified aggregates. This increase was more pronounced for the WPI aggregates compared to the β-Lg aggregates, probably because more protein is aggregated in the WPI solution after heating (**Figure 3**). The more extensively modified aggregates have sizes that are comparable before and after gelation. As discussed earlier the observed increase in size of the aggregates after succinylation can be explained by an increase in voluminosity. Apparently, after gelation at low pH, the increase in voluminosity due to succinylation disappeared and the original size of the aggregates was observed. This might be related to the abolishment of electrostatic repulsion within the aggregates due to the protonated state of the carboxylic acid groups. Together, the results from agarose electrophoresis and DLS experiments strongly suggest that the contribution of disulfide bonds in gels formed in the low pH region (<3) are negligible. In this respect, our results are comparable to those of Otte et al. (*27*), who showed that formation of disulfide bonds does not occur during heat-induced β-Lg gelation at pH 3.

Instead of making protein aggregates more acidic, it seemed also possible to prepare less negatively charged aggregates by chemical modification. Preliminary experiments, where carboxylic groups of aggregates were methylated, essentially according to Hoare and Koshland (*28*), resulted in a shift of the pH of gelation of the modified aggregates toward the more alkaline pH region. On the basis of these preliminary results with succinylated aggregates, this indicates that the methylated aggregates have obtained an increased iso-electric point.

CONCLUSIONS

Our data show that reduction of the electrostatic repulsion of the aggregates (net electric charge) is the driving force for pH-induced gelation. By chemical modification of β-Lg aggregates via succinylation of primary amino groups or methylation of carboxylic acid groups, it is possible to decrease as well as to increase the pH regions of gelation as compared to that of the nonmodified aggregates. This allows the formation of pH-induced gels in the region from pH 2.5 to 9, as compared to pH 4 to 5 for the nonmodified aggregates. In addition to the purified β-Lg, which was used as a model system, we also demonstrated that the same principles hold for WPI.

We also showed that the formation of additional disulfide bonds depends on the pH of gelation and that this has a clear effect on the properties of the gel. Characterization of the gels formed at low pH (2.5−3.5) revealed that disulfide bonds between aggregates are not formed under these conditions. Because the formation of such cross-links is an important factor in determining the final mechanical properties of the gel, control of these mechanical properties via fixation of the iso-electric point of the protein aggregates becomes possible. For application in foodstuffs, however, food-grade alternatives for changing the iso-electric point of the aggregates should be developed

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LITERATURE CITED

(1) Kilara, A.; Sharkasi, T. Y. Effects of temperature on food proteins and its implications on functional properties. *Crit. Rev. Food Nutr.* **1986**, *23*, 323−395.

(2) Oakenfull, D.; Pearce, J.; Burley, R. W. Protein gelation. In *Food Proteins and Their Applications*; Damodaran, S., Paraf, A., Eds; Dekker: New York, 1997.

(3) Bryant, S.; McClements, D. J. Molecular basis of protein functionality with special consideration of cold-set gels derived from heat-denaturated whey. *Trends Food Sci. Technol.* **1998**, *9*, 143−151.

(4) Alting, A. C.; Hamer, R. J.; de Kruif, C. G.; Visschers, R. W. Formation of disulfide bonds in acid-induced gels of preheated whey protein isolate. *J. Agric. Food Chem.* **2000**, *48,* 5001−5007.

(5) De Kruif, C. G. Skim milk acidification. *J. Colloid Interface Sci.* **1997**, *185*, 19−25.

(6) Vreeker, R.; Hoekstra, L. L.; den Boer, D. C.; Agterhof, W. G. M. Fractal aggregation of whey proteins. *Food Hydrocolloids* **1992**, *5*, 423−435.

(7) Barbut, S.; Foegeding, E. A. Ca^{2+}-induced gelation of preheated whey protein isolate. *J. Food Sci.* **1993**, *58*, 867−871.

(8) Sato, K.; Nakamura, M.; Nishiya, T.; Kawanari, M.; Nakajima, I. Preparation of a gel of partially heat-denaturated whey protein by proteolytic digestion. *Michwissenschaft* **1995**, *50*, 389−392.

(9) Elofsson, C.; Dejmek, P.; Paulsson. M.; Burling, H. Characterization of a cold-gelling whey protein concentrate. *Int. Dairy J.* **1997**, *7*, 601−608.

(10) Ju, Z. Y.; Kilara, A. Gelation of pH-aggregated whey protein isolate solution induced by heat, protease, calcium salt, and acidulant. *J. Agric. Food Chem.* **1998**, *46*, 1830−1835.

(11) Ju, Z. Y.; Kilara, A. Effects of preheating on properties of aggregates and of cold-set gels of whey protein isolate. *J. Agric. Food Chem.* **1998**, *46*, 3604−3608.

(12) Roefs, S. P. F. M.; De Kruif, C. G. A model for the denaturation and aggregation of bovine β-lactoglobulin. *Eur. J. Biochem.* **1994**, *226*, 883−889.

(13) Hoffmann, M. A. M.; Van Mil, P. J. J. M. Heat-induced aggregation of β-lactoglobulin: role of the free thiol group and disulphide bonds. *J. Agric. Food Chem.* **1997**, *45*, 2942−2948.

(14) Verheul, M.; Roefs, S. P. F. M.; de Kruif, C. G. Kinetics of heat-induced aggregation of β-lactoglobulin. *J. Agric. Food Chem.* **1998**, *46*, 896−903.

(15) Barbut, S. Effects of calcium level on the structure of preheated whey protein isolate gels. *Lebensm.−Wiss. Technol.* **1995**, *28*, 598−603.

(16) Barbut, S. Relationships between optical and textural properties of cold-set whey protein gels. *Lebensm.−Wiss. Technol.* **1997**, *29*, 590−593.

(17) Matsudomi, N.; Rector, D.; Kinsella, J. E. Gelation of bovine serum albumin and β-lactoglobulin: effects of pH, salts and thiol reagents. *Food Chem.* **1991**, *40*, 55−69.

(18) Wang, C.-H.; Damadoran, S. Thermal gelation of globular proteins: Influence of protein conformation on gel strength. *J. Agric. Food. Chem.* **1991**, *39*, 433−438.

(19) De Jongh, H. H. J.; Groneveld, T.; De Groot, J. Mild isolation procedure discloses new protein structural properties of β-lactoglobulin. *J. Dairy Sci.* **2001**, *84*, 562−571.

(20) Tuinier, R.; Dhont, J. K. G.; De Kruif, C. G.. Depletion-induced phase separation of aggregated whey protein colloids by an exocellular polysaccharide. *Langmuir* **2000**, *16*, 1497−1507.

(21) Verheul, M.; Roefs, S. P. F. M. Structure of whey protein gels, studied by permeability, scanning electron microscopy and rheology. *Food Hydrocolloids* **1998**, *12*, 17−24.

(22) Klotz, I. M. Succinylation. *Methods Enzymol.* **1967**, *11*, 576−581.

(23) Schmidt, D. G.; van Markwijk, B. W. Enzymatic hydrolysis of whey proteins. Influence of heat treatment of α-lactalbumin and β-lactoglobulin on their proteolysis by pepsin and papain. *Neth. Milk Dairy J.* **1993**, *47*, 15−22.
(24) Ellman, G. L. Tissue sulfhydryl groups. *Arch. Biochem. Biophys*. **1959**, *82*, 70−77.
(25) Hegenauer, J.; Ripley, L.; Nace, G. Staining acidic phosphoproteins (phosvitin) in electrophoretic gels. *Anal. Biochem*. **1977**, *78*, 308−311.
(26) Hoffmann, M. A. M.; Roefs, S. P. F. M.; Verheul, M.; van Mil, P. J. J. M.; De Kruif, C. G. Aggregation of β-lactoglobulin studied by *in situ* light scattering. *J. Dairy Sci.* **1996**, *63*, 423−440.
(27) Otte, J.; Zakora, M.; Qvist, K. B. Involvement of disulfide bonds in bovine β-lactoglobulin B gels set thermally at various pH. *J. Food Sci.* **2000**, *65*, 384−389.
(28) Hoare, D. G.; Koshland, D. E., Jr. A procedure for the selective modification of carboxyl groups in proteins. *J. Am. Chem. Soc*. **1966**, *88*, 2057−2058.

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