Formation of Disulfide Bonds in Acid-Induced Gels of Preheated Whey Protein Isolate

Arno C. Alting,*[,†,‡] Rob J. Hamer,[†,§] Cees G. de Kruif,[†,‡,#] and Ronald W. Visschers[†,‡]

Wageningen Centre for Food Sciences, Wageningen, The Netherlands; NIZO Food Research, Ede, The Netherlands; Wageningen University, Wageningen, The Netherlands; and Debye Institute, University of Utrecht, Utrecht, The Netherlands

Cold gelation of whey proteins is a two-step process. First, protein aggregates are prepared by a heat treatment of a solution of native proteins in the absence of salt. Second, after cooling of the solution, gelation is induced by lowering the pH at ambient temperature. To demonstrate the additional formation of disulfide bonds during this second step, gelation of whey protein aggregates with and without a thiol-blocking treatment was studied. Modification of reactive thiols on the surface of the aggregates was carried out after the heat-treatment step. To exclude specific effects of the agent itself, different thiol-blocking agents were used. Dynamic light scattering and SDS−agarose gel electrophoresis were used to show that the size of the aggregates was not changed by this modification. The kinetics of gelation as determined by the development of pH and turbidity within the first 8 h of acidification were not affected by blocking thiol groups. During gelation, formation of large, covalently linked, aggregates occurred only in the case of unblocked WPI aggregates, which demonstrates that additional disulfide bonds were formed. Results of permeability and confocal scanning laser microscope measurements did not reveal any differences in the microstructure of networks prepared from treated or untreated whey protein aggregates. However, gel hardness was decreased 10-fold in gels prepared from blocked aggregates. Mixing different amounts of blocked and unblocked aggregates allowed gel hardness to be controlled. It is proposed that the initial microstructure of the gels is primarily determined by the acid-induced noncovalent interactions. The additional covalent disulfide bonds formed during gelation are involved in stabilizing the network and increase gel strength.

Keywords: *Cold gelation; disulfide bonds; whey proteins; thiol blockers; whey protein isolate*

INTRODUCTION

Most food protein gels are formed during heating and are therefore referred to as heat-induced or heat-set gels. These gels can be prepared from a wide variety of proteins (Oakenfull et al., 1997). For a relatively small number of proteins an alternative method for the preparation of gels at ambient temperature has been reported [reviewed by Bryant and McClements (1998)]. In this so-called cold gelation method, the proteins are first converted into small soluble aggregates by a heating step. Upon cooling, the aggregates remain soluble and no gelation occurs. Gelation can then be induced at room temperature by changing the solvent quality (e.g., by lowering the pH via the addition of sodium chloride or calcium chloride), by the addition of proteases (Sato et al., 1995), or by additional processing such as freezing (U.S. Patent 5,011,702). Cold gelation of heated protein solutions has been reported for β-lactoglobulin, whey protein concentrates, and whey protein isolates (Vreeker et al., 1992; Barbut and Foegeding, 1993; Sato et al., 1995; Elofsson et al., 1997; Ju and Kilara, 1998a,b). Because cold gelation can occur after addition to a food matrix, it has considerable potential in the food industry. The use of whey proteins or other industrial proteins without the need to heat the final product is an attractive alternative for current thickening ingredients (Bryant and McClements, 1998).

The mechanical properties of both cold- and heat-set gels depend on the protein composition and concentration, their interaction with other ingredients, and the preparation technique. For heat-set gels a wealth of information is available on the mechanism and kinetics of the aggregation (Roefs and de Kruif, 1994; Hoffmann and van Mil, 1997; Ikeda et al., 1999) and gelation process (Oakenfull et al., 1997; Verheul et al., 1998a). Several suggestions for scaling laws that relate molecular properties of ingredients to macroscopic properties of the gels have been made (Oakenfull and Scott, 1986; Wang and Damodaran, 1990). The formation of disulfide bonds in heat-set gels is well established (Hoffmann and van Mil, 1997). A smaller number of papers have been published that relate the mechanical properties of cold-set gels to specific preparation conditions (Ju and Kilara, 1998a,b) and properties of ingredients such as the presence of calcium (Barbut and Foegeding, 1993; Barbut, 1995, 1997). Despite these studies there are still a number of unanswered questions regarding the cold gelation process. One of these questions concerns the molecular events that lead to gelation of the reactive aggregates. In particular, no study has been made of

* Address correspondence to this author at NIZO Food Research, P.O. Box 20, 6710 BA Ede, The Netherlands [telephone +31 (0)318 659571; fax +31 (0)318 650400; e-mail alting@nizo.nl].

† Wageningen Centre for Food Sciences.

‡ NIZO Food Research.

§ Wageningen University.

Debye Institute.

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the role of noncovalent interactions versus covalent chemical reactions at this stage of the process. However, the common notion is that noncovalent interactions play a dominant role.

In contrast to heat-induced gelation, in which aggregation and gelation are intertwined, the two processes can be studied separately in a cold gelation procedure. In the first step it is possible to control and manipulate the properties of the aggregates by different heating strategies or chemical treatments before heating (Elofsson et al., 1997; Hongsprabhas and Barbut, 1997; Ju and Kilara, 1998b). In the second step it is possible to study how the properties of the aggregates influence the gelation process. Control of the gelation process by modification of the aggregates after the heating step has not been reported yet. This approach has the obvious advantage that only effects on the actual gelation process are studied because the heating step is carried out under identical conditions. This paper demonstrates that with this approach it is possible to study specific aspects of the gelation mechanism. It appears that disulfide bonds are formed in the second stage of the process of cold gelation and that they have an influence on the final mechanical properties of the gels. Through changing the amount of reactive thiol groups on the aggregates after heating, it is possible to control gel hardness, without changing the amount of ingredients.

MATERIALS AND METHODS

Reagents and Chemicals. Glucono-δ-lactone (GDL), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), sodium dodecyl sulfate (SDS), dithiothreithol (DTT), iodoacetamide (IAA), *p*-chloromercuribenzoic acid (PCMB), and *N*-ethylmaleimide (NEM) were obtained from Sigma Chemical Co. (St. Louis, MO). Electrophoresis grade agarose was obtained from Life Technologies (Paisley, Scotland). Phastgel blue R tablets were from Pharmacia Biotech (Uppsala, Sweden). The whey protein isolate (WPI) Bipro was obtained from Davisco Foods International Inc. (La Sueur, MN).

Preparation of Reactive WPI Aggregates. WPI was dissolved in double-distilled water at a concentration of 9% (w/w) and stirred for at least 2 h (Verheul and Roefs, 1998). The WPI solution was centrifuged (30 min, 19000*g*, 20 °C), and the supernatant was filtered (0.45 μm; Millex-SV, Millipore Corp., Bedford, MA). Reactive WPI aggregates (9%, w/w) were prepared by heating the WPI solution (300 mL) in a water bath for 2 h at 68.5 °C (Tuinier et al., 2000) and cooling with running tap water. The amount of native proteins after the heat treatment was determined with a standard assay involving acid precipitation and gel permeation chromatography (Hoffmann et al., 1996). The solution of WPI aggregates was diluted with filtered (0.22 μm; Millex-GV, Millipore Corp.) double-distilled water to a concentration of 2% (w/w) and stored at 4 °C. Sodium azide (0.02% final concentration) was added as a preservative.

Blocking of the Reactive Thiol Group. Reactive aggregates (2%, w/w) were treated with three different reagents, NEM, IAA, and PCMB, at various concentrations (0−5 mM). After the addition of the thiol-blocking agents, the reaction was allowed to proceed for at least 30 min at room temperature before further experiments were started. In some experiments the excess of reagent was removed by dialysis.

Accessibility of the Thiol Groups. Accessible thiol groups before and after treatment with sulfhydryl reagents were determined using Ellman's reagent (Ellman, 1959) essentially as described by Hoffmann and Van Mil (1997), except a Bis-Tris/HCl buffer (pH 7) was used instead of a Tris/HCl buffer. The assay was performed in the absence of detergents such as urea or SDS, because under these conditions only the thiol groups of interest, those on the surface of the aggregates, were determined. The concentration of the different thiol-blocking agents at which no further decrease in extinction at 412 nm was observed was determined.

Preparation and Solubilization of Acid-Induced Gels. GDL was added to the 2% (w/w) degassed WPI solution to induce cold gelation. The total amount of GDL added depends on the protein concentration (De Kruif, 1997). Typically, such an amount of GDL (0.15%) was added that at ambient temperature the pH of the solution was gradually lowered from pH 7.2 to a pH of ∼5 (after ∼24 h). This acidification induced gelation of the WPI solution. Gel samples were mixed with a buffer containing SDS (see Agarose Gel Electrophoresis) to solubilize the gels. Alternatively, larger amounts of GDL were added. We observed that the gel system becomes soluble again at pH <4.

Agarose Gel Electrophoresis. SDS−agarose continuous gel electrophoresis (0.4% agarose) was performed to determine the differences in molecular weight of the different treated WPI aggregates. The electrophoresis buffer consists of 100 mM Tris, 50 mM sodium acetate, 2 mM EDTA, and 0.1% SDS and was brought to pH 7.9 with concentrated acetic acid. Aggregates (in solution or gel) were mixed with 3 parts of 20 mM Bis-Tris buffer (pH 7.0) and 5% SDS and were held at ambient temperature with constant stirring. After overnight incubation, no gel particles could be observed with a standard microscope (at 400× magnification). In some experiments a treatment with the disulfide reducing agent DTT (0.05%) was carried out to break all of the disulfide bonds. 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 30 V for ∼3 h and stained with Phastgel blue R (Pharmacia Biotech, Uppsala, Sweden).

Dynamic Light Scattering (DLS) Experiments. DLS experiments were performed as outlined by Verheul et al. (1998b). 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 Corp.).

Turbidity Measurements. Turbidity measurements were performed at 25 °C on a Cary 1E UV−vis spectrophotometer (Varian Nederland BV) equipped with a temperature controller. The turbidity was measured in time as the absorbance at 500 nm with the pH being monitored simultaneously. Samples were measured in quartz cuvettes with a path length of 2 mm.

Permeability Measurements. Permeability measurements were performed as extensively described by Verheul and Roefs (1998). Briefly, gels were prepared from 2% WPI aggregates with and without a pretreatment with NEM at ambient temperature in glass tubes (25 cm × 3.7 mm) with open ends. Approximately 24 h after the addition of GDL, they were placed in a measuring device to monitor the flow of solvent under hydrostatic pressure (9×10^3 Pa m^{-1}). Permeability coefficients were determined by measuring the flux of solvent in time.

Gel Hardness. Gel hardness was determined by a texture analyzer (type TA-XT2, Stable Micro Systems ltd., Godalming, U.K.). GDL (0.15%, w/w) was added to 100 g of a 2% solution of WPI aggregates, and gels were formed in a beaker at ambient temperature. Approximately 24 h after the addition of GDL, the acid-induced gels were penetrated with a wire mesh device. The mesh consisted of four blades (45 × 1.5 × 2 mm) of stainless steel arranged in a double cross. A force−time curve was obtained at a crosshead speed of 0.3 mm/min for a 10 mm displacement, and gel hardness was expressed as the force (g) at the maximum peak of the force−time curve (Bourne, 1978).

Confocal Scanning Laser Microscopy. Imaging was performed using a Leica confocal scanning laser microscope, type TCS-SP, configured with an inverted microscope, and an ArKr laser for single-photon excitation. The protein gels were stained by applying 2 μL of an aqueous solution of 0.05% Rhodamine B. The 568 nm laser line was used for excitation inducing a fluorescent emission of Rhodamine B, detected between 600 and 700 nm.

RESULTS AND DISCUSSION

Heat treatment of a WPI solution (9%) resulted in the formation of reactive WPI aggregates with an approximate hydrodynamic diameter of 80 nm (Table 1) with in excess of 95% of the native proteins participating in aggregate formation. The heated solution was diluted to a concentration of 2% (w/w) and stored at 4 °C for not longer than 3 days, during which time there were no significant changes in the hydrodynamic diameter of the aggregates. An initial protein concentration of 9% was chosen because this enables us to study gel characteristics depending on the protein concentration.

A second type of aggregates was prepared by adding agents that block the free thiol groups present on the reactive WPI aggregates. To eliminate any specific effect of the agent itself, three different types were used: IAA, PCMB, and NEM. The lowest concentrations of the reagents at which no further decrease of the absorption in the Ellman's assay occurred was determined (0.5 mM for NEM and PCMB; 2 mM for IAA). At this concentration it is justifiable to assume that all of the accessible thiol groups are blocked. Differences in concentration of the agents are presumably due to differences in specificity and in the conditions for optimal activity of the blocking agents (Wong, 1991). The treatment with thiol-blocking agents did not change the hydrodynamic diameter of WPI aggregates, as determined by DLS (Table 1).

A 2% (w/w) solution of Bipro contains ~0.75 mM β-lactoglobulin. Bovine β-lactoglobulin contains a total of five cysteine residues, four of which are involved in intramolecular disulfide bonds (Swaisgood, 1982). After the heat-induced aggregation, we estimate, using ϵ (412 nm) = 13600 M^{-1} cm^{-1} of 2-nitro-5-mercaptobenzoic acid, that the solution still contained 0.3 mM accessible thiol groups. Thus, in accordance with the model of Roefs and De Kruif (1994), a substantial fraction of the free thiol groups is still accessible on the surface of the aggregates. Both the thiol-blocking reaction and the determination of the accessible thiol groups were performed in the absence of detergents to avoid disruption of the aggregate structure and because only the reactive groups on the surface of the aggregates were of interest for this study. No significant change in the amount of accessible thiol groups was observed during cold storage (up to 3 days).

Addition of salt to or lowering the pH of a solution of either thiol-modified (blocked) or reactive (unblocked) WPI aggregates induces gelation at ambient temperature. Addition of ascorbate or citrate results in an instantaneous decrease of the pH and therefore in an irregular gelation. Addition of GDL, which slowly hydrolyzes to gluconic acid, causes a gradual reduction in pH and a regular gel. The point of gelation strongly depends on pH. Gelation occurred at a minimal protein concentration of 0.5% (9% diluted to 0.5%). The protein concentration applied in this work is 4 times higher. At a pH near the isoelectric point of the proteins (5.1 for β-lactoglobulin), the electrostatic repulsion between the aggregates is reduced and therefore aggregation through noncovalent chemical interactions is promoted (Ju and Kilara, 1998a). The development of the pH and the turbidity, measured as the absorbance at 500 nm, during the first 8 h after the addition of GDL displayed identical kinetics for both types of aggregates. After addition of GDL, the turbidity remains constant for approximately the first 3 h. Thereafter, a rapid increase of the turbidity occurs that marks the initial formation of the network. An absorbance of 0.1 was reached at the same pH (within 0.1 pH unit) for both types of aggregates.

Table 1. Effect of Cold Gelation Process and Thiol-Blocking Agents on the Hydrodynamic Diameter and Scattering Intensity of WPI Aggregates[a]

	intensity (cps × 1000)	diameter (nm)
Aggregates before Gelation		
control	78	83.2 ± 0.8
0.5 mM NEM	89	81.3 ± 0.8
2 mM IAA	89	81.3 ± 0.7
0.5 mM PCMB	92	81.4 ± 1.1
Aggregates after Gelation and Resolubilization		
control	302	288 ± 4.0
0.5 mM NEM	96	81.5 ± 0.9
2 mM IAA	147	114.5 ± 1.1
0.5 mM PCMB	103	83.8 ± 1.1

[a] Aggregates and gels were dissolved in SDS buffer system (1:3) (final protein concentration = 0.5%). Errors represent the standard error of the cumulant fits within one measurement.

The microstructure of the gels was characterized by permeability measurements and CSLM. In heat-set gels of WPI the permeability coefficient is a good parameter for testing gel structure (Verheul and Roefs, 1998). Application of this technique to describe cold-set gels is new. The permeability coefficients of the cold-set gels of blocked and unblocked WPI aggregates did not differ significantly (1.1×10^{-14} m^2) but were lower than those of heat-set WPI gels with the same protein concentration (Verheul and Roefs, 1998). CSLM measurements confirmed that the microstructures of the gels were quite similar (Figure 1). Because both the microstructure and the initial kinetics of gelation are not perturbed by modifying free thiol groups on the surface of the aggregates, it is concluded that, under the conditions applied, the initial morphology of the network is established by noncovalent interactions.

After 24 h of incubation with GDL, gels were solubilized, and very clear differences between the two types of aggregates were observed. Gels that were prepared from aggregates of which the thiol groups were blocked dissolved rapidly within 15 min. Gels that were made from nontreated WPI aggregates dissolved much more slowly. It took several hours (overnight) before gel particles were no longer visible using a laboratory microscope. Formation of large aggregates and a possible effect of a slight excess of thiol-blocking agent during the solubilization are ruled out, because it took place at pH 7, at which the aggregates are stable. Also, formation of large aggregates was not observed in material that had not been acidified and gelated, but was dissolved directly in SDS buffer.

Agarose gel electrophoresis, in the presence of SDS, was used to demonstrate differences in electrophoretic mobility of the WPI aggregates (Figure 2). Because the agarose electrophoresis gels were run without stacking gel, diffuse bands were observed. Treatment with dif-

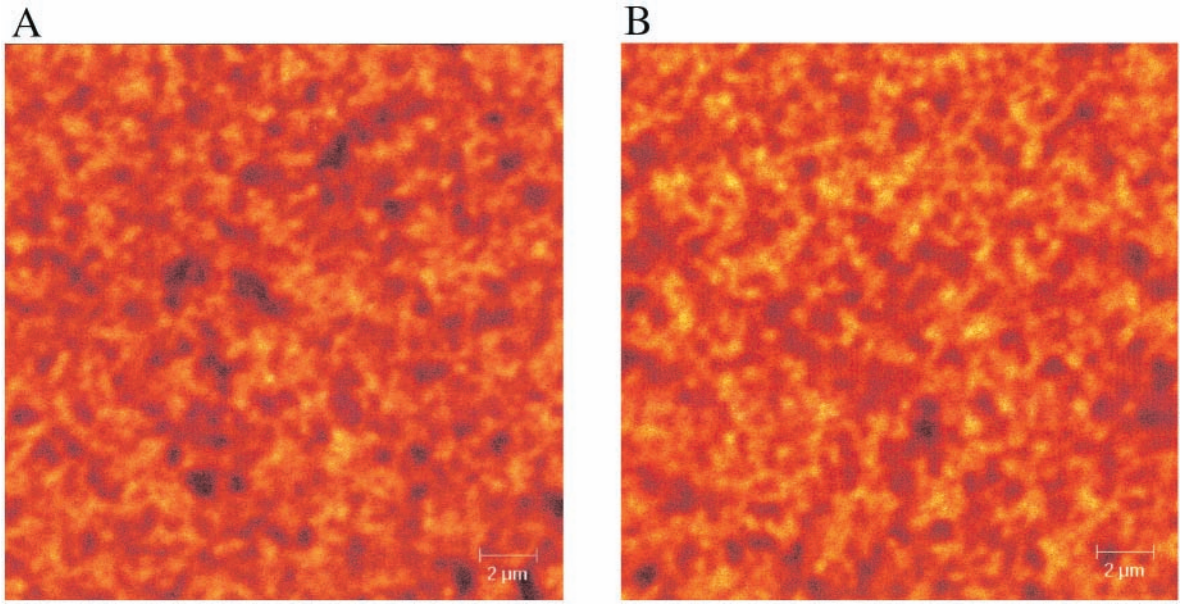

Figure 1. CSLM images of 2% WPI gels (A), and gels of WPI after a treatment with NEM (B). A fluorescent dye, Rhodamine B, was applied to the gels. The dye binds noncovalently to the protein network. Light areas in the images reflect higher dye concentrations and therefore represent the protein network. Dark areas represent aqueous pores.

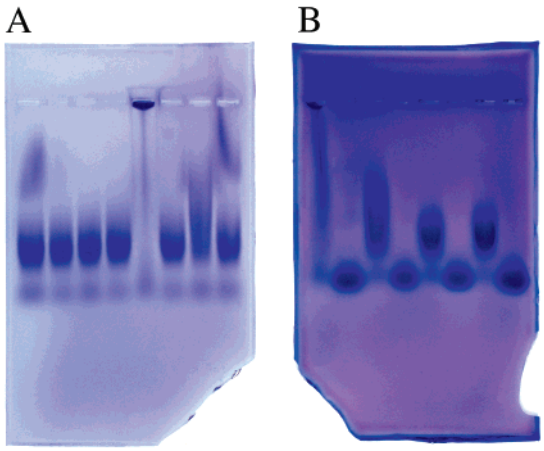

Figure 2. (A) SDS−agarose gel electrophoresis of WPI aggregates: effect of thiol-blocking agents on the molecular weight of the aggregates before and after gelation and resolubilization. The four lanes on the left-hand side of the gel contain the different aggregates before gelation: without thiol-blocking agent (1); and treated with NEM (2), IAA (3), and PCMB (4). The four lanes on the right-hand side contain the aggregates after gelation: without thiol-blocking agent (5); and treated with NEM (6), IAA (7), and PCMB (8). The formation of large aggregates in lane 5 is indicated by the upper arrow. The spot in lane 1 is caused by nonspecific staining. (B) Effect of DTT on the molecular weight of the aggregates before and after gelation and resolubilization. The aggregates after gelation and dissolution are represented in lanes 1−4. Lanes 1 and 2 show the aggregates without thiol-blocking treatment before (1) and after (2) the addition of DTT. Lanes 3 and 4 show the aggregates treated with NEM before (3) and after (4) the addition of DTT. Lanes 5−8 contain the aggregates before gelation. Lanes 5 and 6 contain the aggregates without thiol-blocking treatment before (5) and after (6) the addition of DTT. Lanes 7 and 8 show the aggregates treated with NEM before (7) and after (8) the addition of DTT. As indicated by the lower arrow, the electrophoretic mobilities of all aggregates are the same after treatment with DTT.

ferent thiol-blocking agents had no influence on the migration of the WPI aggregates (Figure 2A, lane 1−4). However, cold gelation had a large effect on the mobility of some of the aggregates. After gelation, the aggregates without thiol-blocking treatment hardly entered the electrophoresis gel (Figure 2A, lane 5). Blocking the accessible thiol groups on the aggregates before starting gelation prevented the formation of such large aggregates (Figure 2A, lanes 6−8), and the mobility did not differ from that of the aggregates prior to gelation. This effect was evident with all types of thiol-blocking agents used. Another indication that disulfide bonds are formed during the cold gelation process is presented in Figure 2B. Addition of DTT, an agent that reduces disulfide bonds, has a clear effect on the size of the aggregates. Both types of aggregates were broken down further to fragments of approximately equal electrophoretic mobilities.

The size of the aggregates before and after gelation was also determined by DLS. As discussed, the sizes of both type of aggregates were very similar before gelation (Table 1). In agreement with the results from the electrophoresis experiments, a clear increase in hydrodynamic diameter after cold gelation and solubilization occurred only with the reactive WPI aggregates. This effect was not observed with the other type of aggregates. Formation of larger aggregates was prohibited by the thiol-blocking treatment, independent of the type of thiol-blocking agent. In all cases, addition of 0.5% DTT caused a dramatic decrease of the intensity and reliable light-scattering measurements were not possible.

Mechanical properties of the gels formed from the two types of aggregates were characterized by determining gel hardness. In the case of NEM-treated aggregates it was necessary to remove excess blocking agent by dialysis prior to gelation. The most substantial increase of the hardness of the gel takes place during the first 24 h. For this reason, in all experiments gels were characterized after 24 h of incubation with GDL. A force−time curve was obtained, and gel hardness was expressed as the force (g) at the maximum peak of the force−time curve (Bourne, 1978). To correct for small differences between experiments caused by variation in ambient temperature, the relative gel hardness was plotted against the percentage aggregates of which the

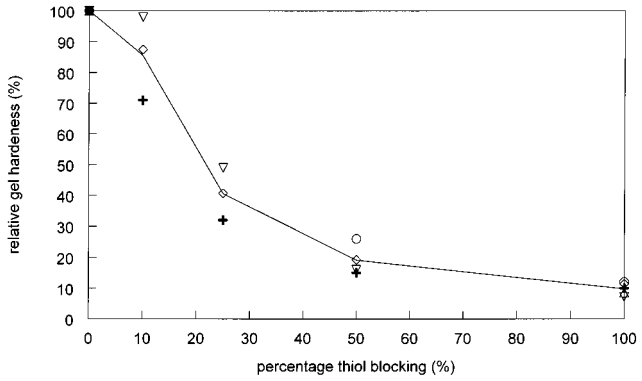

Figure 3. Effect of the amount of thiol-blocking (NEM) on the relative gel hardness of acid-induced gels. Results of the different experiments are represented as different symbols. The solid line connects the average values of the experiments.

Table 2. Effect of Cold Gelation Process and Thiol-Blocking Agents on the Hydrodynamic Diameter and Scattering Intensity of WPI Aggregates as a Function of the GDL Concentration[a]

[GDL] (%)	intensity (cps × 1000)	diameter (nm)
	Aggregates before Gelation	
0	293	83.2 ± 0.8
	Aggregates after Gelation and Resolubilization	
0.7	1650	277 ± 2.6
0.8	900	122 ± 1.0
0.9	680	93 ± 1.1
1.0	597	85 ± 0.6
1.1	535	80 ± 0.7
1.2	505	78 ± 0.9
1.3	465	76 ± 1.1

[a] Final protein concentration = 2%. Errors represent the standard error of the cumulant fits within one measurement.

accessible thiol groups were blocked. Blocking the accessible thiol groups on the WPI aggregates significantly decreased (5−10-fold) the gel hardness of the gels formed. This effect was independent of the type of thiol-blocking agent used (results not shown). In the case of unblocked aggregates the hardness of the gel continued to increase slowly for a few days. This effect was not observed for the gelation of WPI aggregates treated with a thiol-blocking agent (NEM). Remarkably, it was also possible to vary gel hardness by preparing mixtures of the two types of aggregates, thus gradually varying the ability to form disulfide bonds (Figure 3). The resulting gels had intermediate hardness, which could be controlled with the ratio of the two types of aggregates in the mixture. DLS and agarose gel electrophoresis results showed that after gelation of these mixtures, aggregates with an intermediate size were formed (results not shown).

It was also observed that by adding larger amounts of GDL it was possible to start with a solution of aggregates, reach an intermediate gel structure, and end up with a redissolved solution of aggregates (pH <4). Table 2 shows that the formation of larger aggregates depends on the amount of GDL added. Addition of relatively small amounts of GDL prolongs the time during which the system is a gel, thereby increasing the exposure time at which repulsive electrostatic interactions are minimal (isoelectric point). Under the conditions applied, the rate of disulfide bond formation is probably much faster in the gel compared to the rate in solution. During this "gel" period covalent bonds can be formed, and this will lead to the formation of larger aggregates. Thus, it seems likely that the formation of disulfide bonds between the aggregates is facilitated by the acid-induced noncovalent interactions and the amount of disulfide bonds formed depends on the time during which the system is in the gel state.

Several papers have reported on the effect of blocking the free thiols during the heat and cold gelation of whey proteins. In all cases the thiol-blocking agent was added prior to the heating step. Hoffmann and van Mil (1997) demonstrated that the thiol group plays a crucial role in the heat-induced aggregation of β-lactoglobulin. Hongsprabhas and Barbut (1997) investigated the effect of the thiol-blocking agent NEM during the heating step of cold gelation, when the reactive aggregates are formed. They also concluded that disulfide bonds are mainly involved in the polymerization step prior to gelation. This is in agreement with the statement of Wang and Damodaran (1990), who investigated thermal gelation of globular proteins. They reported that the role of disulfide bonds in gelation is to increase the weight-average molecular weight of the proteins rather than to form a specific network in the gel. Also, Ju and Kilara (1998b) found that the hardness of cold-set gels increased with the concentration of protein during preheating and attributed this to an increase in the size of the aggregates. In the above studies it is difficult to discriminate between effects of disulfide bond formation on the formation of aggregates or on the formation of a specific network because it is not clear if the aggregates are still reactive during (cold) gelation.

We have taken a new approach to distinguish between effects on the formation of aggregates and effects on the formation of a specific network. It was possible to study the formation of disulfide bonds during the gelation process by adding thiol blockers after the first heating step. It was shown, by electrophoresis and DLS, that the size of the reactive aggregates was not significantly changed by the thiol-blocking reactions. In our experiments the only variable was the amount of accessible thiol groups. Our results demonstrate that during cold gelation, disulfide bonds are formed, resulting in larger covalently linked aggregates. This is surprising because the formation of disulfide bonds, either as a result of an oxidation reaction or from thiol−disulfide interchanges, normally occurs under alkaline conditions (Bryant and McClements, 1998) and lowering the pH would only slow these reactions. We attribute the formation of disulfide bonds under these conditions to a large increase of the effective concentration. As a result of the noncovalent interactions the free thiol groups and the disulfide bonds are very close and the formation of disulfide bonds is promoted. The formation of disulfide bonds increases the molecular weight of the aggregates formed during gelation and is involved in stabilizing the network, with a concomitant increase in gel strength.

To the authors' knowledge, this is the first work that describes the effect of thiol-blocking agents on the second stage of the process of cold gelation of WPI and on the mechanical properties of the gels. Recently, Hirano et al. (1999) observed changes in the hardness of acid milk gels caused by the addition of hypothiocyanite ion ($OSCN^-$) and hydrogen peroxide (H_2O_2). They concluded that the decrease in the hardness of the acid milk gel resulted from the decrease of thiol groups in milk proteins in the presence of $OSCN^-$ or H_2O_2, which inhibited the intermolecular disulfide formation re-

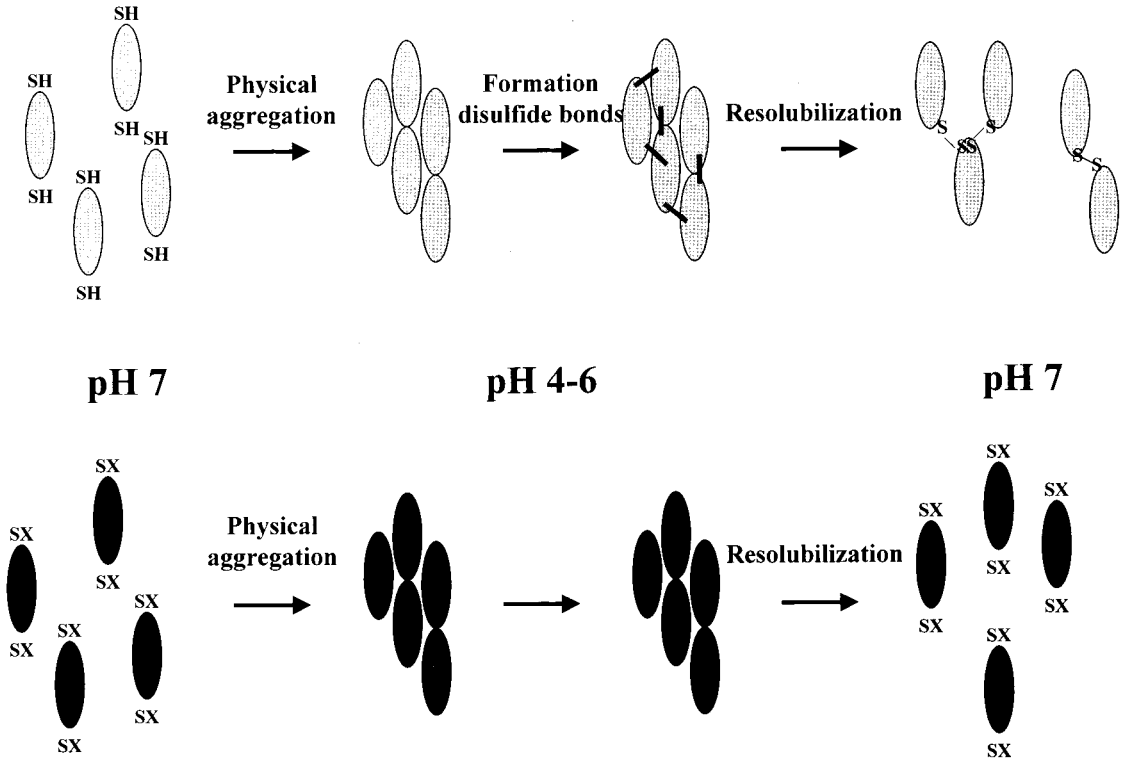

Figure 4. Model for the formation of intermolecular disulfide bridges and their role during the acid-induced cold gelation of heat-treated whey proteins. The oval shapes represent the WPI aggregates after heating (size ~80 nm). The upper part describes the gelation process of unblocked aggregates. In this case the free thiol groups (SH) can form disulfide bonds after noncovalent chemical aggregation, and resolubilization yields relatively large aggregates. The lower part indicates that when the thiol groups are blocked (SX), no disulfides can be formed. After resolubilization, the size of these aggregates is not affected by the gelation process.

quired for gelation. This is consistent with our observation that a relationship exists between the amount of disulfide bonds formed during gelation and the hardness of the gel. No differences were observed in the microstructure of the gels, and no indications were found that the noncovalent interactions had changed as a result of the thiol-blocking treatment. On the basis of these results we propose a model for the formation and involvement of disulfide bonds in the second stage of the cold gelation process (Figure 4). When solutions of aggregates that differ in the amount of accessible thiol groups are gradually acidified, the noncovalent aggregation mechanism is not different and the same microstructures are apparently formed. The acid-induced noncovalent interactions between the aggregates facilitate covalent chemical reactions, leading to the formation of disulfide bonds only when free thiol groups are present. The amount of disulfide bonds formed depends on the amount of free thiol groups available. This opens the opportunity to control the hardness of the gels through mixing proteins with different amounts of surface-exposed free thiol groups. With this new insight it becomes possible to directly control the hardness of cold-set gels without changing the amount of ingredients, making application of the technique more feasible.

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LITERATURE CITED

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

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

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

Bourne, M. C. Texture profile analysis. *Food Technol.* **1978**, *3*, 62−66, 72.

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

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

Ellman, G. L. Tissue sulfhydryl groups. *Arch. Biochem. Biophys.* **1959**, *82*, 70−77.

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

Hirano, R.; Hirano, M.; Hatanaka, K. Changes in hardness of acid milk gel by addition of hypothiocyanite ion, hydrogen peroxide and their effects on sulfhydryls in milk proteins. *J. Jpn. Soc. Food Sci. Technol.* **1999**, *46*, 376−381.

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.

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 Res.* **1996**, *63*, 423−440.

Hongsprabhas, P.; Barbut, S. Effects of *N*-ethylmaleimide and $CaCl_2$ on cold gelation of whey protein isolate. *Food Res. Int.* **1997**, *30*, 451−455.

Ikeda, S.; Foegeding, E. A.; Hagiwara, T. Rheological study on the fractal nature of the protein gel structure. *Langmuir* **1999**, *15*, 8584−8589.

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.* **1998a**, *46*, 1830−1835.

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.* **1998b**, *46*, 3604−3608.

Oakenfull, D. G.; Scott, A. G. Stabilization of gelatin gels by sugars and polyols. *Food Hydrocolloids* **1986**, *1*, 163−175.

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

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.

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

Swaisgood, H. E. Chemistry of milk proteins. In *Developments in Dairy Chemistry—I*; Fox, P. F., Ed.; Applied Science Publishers: London, U.K., 1982.

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.

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.

Verheul, M.; Roefs, S. P. F. M.; Mellema, J.; De Kruif, C. G. Power law behaviour of structural properties of protein gels. *Langmuir* **1998a**, *14*, 2263−2268.

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

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

Wang, C.-H.; Damodaran, S. Thermal gelation of globular proteins: weight-average molecular weight dependence of gel strength. *J. Agric. Food Chem.* **1990**, *38*, 1157−1164.

Wong, S. S. *Chemistry of Protein Conjugation and Cross-linking*; CRC Press: Boca Raton, FL, 1991.

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