Cold gelation of globular proteins

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CONTENTS

Abstract

Protein gelation in food products is important to obtain desirable sensory and textural properties. Cold gelation is a novel method to produce protein-based gels. It is a two step process in which first thermally induced protein aggregates are prepared by a heat treatment of a solution of native globular proteins at low ionic strength. After cooling, gelation of the dispersion of repulsive aggregates is induced in the second step by lowering the pH or by adding salt at ambient temperature. Cold-set gelation finds applications in food products with a delicate flavor and texture. In addition cold-set gels can be used as a thickening agent or in encapsulation of sensitive materials.

The purpose of the research described in this thesis was to investigate the molecular mechanisms of the acid-induced cold gelation process. Therefore it was studied (i) how different aggregate properties determined the final properties of cold-set whey protein gels, (ii) how structural and rheological properties of the gels scaled with the protein concentration, and (iii) how the final gel properties depended on the use of different cysteine-containing globular proteins.

The results demonstrated that reduction of the electrostatic repulsion initiated the formation of a randomly aggregated protein network by physical interactions. Surprisingly, additional covalent disulfide bonds were formed under the acid conditions. The disulfide bonds stabilized the initial network and increased the mechanical gel strength. The formation of disulfide bonds depended on the number and accessibility of thiol groups and disulfide bonds present in the various protein molecules. Therefore, the disulfide bonds are important control parameters that can be used to tune the texture of (cold-set) gels. In addition, the contour length of the linear-shaped aggregates prepared in the first step affected the mechanical gel strength of cold-set gels. For smaller aggregates percolation is preceded by the formation of clusters, yielding less effective contact points and therefore weaker gels compared to cold-set gels prepared from long fibrillar structures. Moreover, the length of the linear-shaped aggregates determined the appearance of the cold-set gels. Cold gelation is a relevant method for the application of globular proteins as an efficient structuring ingredient in food systems.

Keywords: globular proteins, whey protein, ovalbumin, cold gelation, disulfide bonds, texture, gel hardness

Chapter 1

General introduction

General introduction

Protein gelation is used in many industrial applications. In food products it is important to obtain desirable sensory and textural properties such as in sausages, custards, cheese, yogurt, tofu, and egg-products. The mechanical properties of gels are important in photographic films and medicine capsules. In some lithographic processes a casein-based gel layer is applied to form acid-resistant masks.

The word jelly appeared for the first time in the fourteenth century and was derived, via the French *gelée*, meaning frost, from the Latin *gelare*, meaning to freeze. The scientific term "gel" was introduced by Thomas Graham (>1869), the founding father of colloid chemistry (for a short review see Oakenfull et al., 1997). Many definitions of a gel have since been given in the literature. Recent definitions that are adhered to in this thesis were given by Ziegler and Foegeding (1990), who defined a gel as "a continuous network of macroscopic dimensions immersed in a liquid medium and exhibiting no steady-state flow", and Wong (1989), who defined gelation as "aggregation of denatured molecules with a certain degree of order, resulting in the formation of a continuous network".

Cold gelation processes are those in which proteins or protein particles are made to gel at (sub)ambient temperatures. A prerequisite for cold gelation is a dispersion of "activated" globular protein (particles) which upon a change of conditions form a protein network. The gelation can be induced by lowering the temperature, the addition of salt, a change in pH, the addition of an enzyme or chemical cross-linker, high hydrostatic pressure, or the addition of a non-solvent. Strictly speaking, cheese- and yogurt-making fall within this definition. However, in the literature the term cold gelation is limited to the gelation of non-casein proteins. This type of gelation can be induced at a late stage of production or even in the packaged product and therefore it has applications in structuring food products, masking flavors, and encapsulating specific compounds. This thesis describes the conditions and mechanisms by which acid-induced cold gelation processes of cysteine-containing globular food proteins operate.
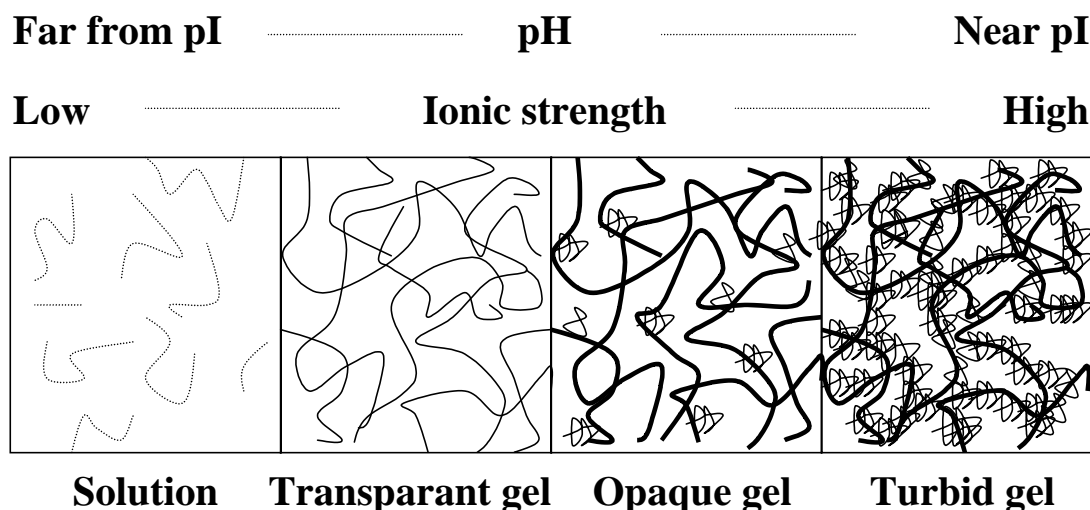
Functionality of globular proteins

The term "functionality" as applied to food ingredients has been defined as "any property besides nutritional attributes that influences an ingredient's usefulness in foods" (Boye et al., 1997). Functional properties of protein ingredients are related to their physical, chemical, and conformational properties (Damadoran, 1997). Therefore, they depend not only on their intrinsic properties but also on their degree of denaturation, or more generally speaking on the changes in native conformation. Denaturation of globular proteins is in most cases a prerequisite to "activate" the functionality that is desired for the sensorial and textural properties of food. Denaturation has been defined as a major change of the very specific native protein structure, without alteration of the amino acid sequence (Tanford, 1968) and is a consequence of an altered balance between the different forces, such as electrostatic interactions, hydrogen bonds, disulfide bonds, dipole-dipole interactions, and hydrophobic interactions, that maintain a protein in its native state. The loss of the globular character of proteins during heating can be primarily attributed to the increased entropy of the unfolded state of the protein (Creighton, 1978) and is in principle reversible. Complete unfolding only occurs in the presence of strong denaturants, such as urea or guanidine hydrochloride. Acid- or heat-induced denatured proteins are usually not completely unfolded and retain most part of their native-like structure (Hermansson, 1978). Forces which are involved in folding and stabilizing the native protein structure are also involved in aggregate formation. An increase in the effective hydrophobicity is an indication of protein unfolding. When too many

hydrophobic sites are exposed, intermolecular interactions between these sites become inevitable and aggregation of protein molecules occurs.

In addition to the noncovalent interactions, intermolecular covalent reactions can also occur on heating in the case of cysteine-containing proteins. Aggregation reactions, and in particular the formation of intermolecular disulfide bonds, may prevent the renaturation of the unfolded protein molecule upon cooling, so that the process becomes irreversible. Therefore, the details of the kinetics of both the unfolding and aggregation of protein molecules are important to obtain the optimal functionality of globular proteins as thickening or gelling agents in foods.

Formation of a protein network

A protein gel consists of a spatial network of protein particles. The functionality is determined by both the spatial distribution of the protein particles and by the contribution of covalent and noncovalent bonds to the network. The relative contributions of these different types of bonds will, in addition to the intrinsic properties of the protein (hydrophobicity, electrostatic interactions, disulfide bonds, molecular mass, and amino acid composition), depend on the conditions applied during gelation (protein concentration, pH, temperature, ionic strength and type of ion, and hydrostatic pressure) (Totosaus et al., 2002; Smith, 1994, Phillips et al., 1994). The resulting size, shape and spatial arrangement of the protein aggregates and their response to deformation can therefore vary widely and have an impact on gel properties like rheological behavior, sensoric quality and water-holding capacity.

Far from pI .... **pH** .... **Near pI**

Low .... **Ionic strength** .... **High**

Solution   **Transparant gel**   **Opaque gel**   **Turbid gel**

Figure 1:   Relation between protein gel appearance and modulation of the electrostatic repulsion. Figure adapted from Doi and Kitabatake (1997).

Tombs (1974) presented two models for heat-induced globular protein gels: random aggregation or aggregation into a "string of beads" structure, resulting in a turbid coarse network or a transparent fine-stranded protein network, respectively. Intermediate structures are also possible. The type of network formed is associated with changes in the balance between attractive and repulsive forces between the aggregating particles (Doi, 1993). **Figure 1** depicts how for example pH and ionic strength influence the final gel properties during heat-induced gelation. At low ionic strength or at pH values far from the iso-electric point (pI) of the active protein, electrostatic repulsive forces hinder the formation of random aggregates and more linear polymers are formed, resulting in transparent and fine-stranded gels. When heat-induced gelation occurs at high ionic strength or at a pH near the pI of the protein, repulsive forces are weaker and denatured proteins

aggregate randomly by physical interactions such as hydrophobic and van der Waals interactions into particulate, turbid gels.

Induction of gelation

Gelation of a solution of globular proteins can be induced in various ways. Heat-induced gelation is the most commonly studied phenomenon in food science, and responsible for the structure present in many everyday heat-set foods (Totosaus et al., 2002). A second type of physically induced gelation is the less common method of hydrostatic-pressure-induced gelation. Both gelation methods are single-step methods. Under the conditions applied, the processes of the denaturation of the protein molecules and subsequent aggregation to a space-filling protein network proceed simultaneously.

In addition, other gelation methods are reported: salt-induced gelation, acid-induced gelation, and enzyme-induced gelation (Totosaus et al., 2002). Enzyme-induced gelation is reported for both protein cross-linking and protein-degrading enzymes, transglutaminase [EC 2.3.2.13] and specific proteolytic enzymes, respectively. Transglutaminase is capable of catalyzing an acyl transfer reaction between lysine and glutamine residues, introducing covalent cross-links between proteins (Zhu et al., 1995). This type of gelation is reported for proteins having a relatively open tertiary structure, such as caseins. Otte et al. (1995) reported that the degradation of whey proteins by a protease specific for glutamic acid and aspartic acid residues resulted in aggregation and gelation. The clotting of blood is also an enzyme-induced gelation process, caused by the action of thrombin on fibrinogen. In this type of gelation the protein molecule is "activated" by the proteolytic action of the enzyme, resulting in the exposure of sites that can interact with other activated protein molecules.

Salt- or acid-induced types of gelation consist of two steps. Direct addition of acid or salt usually does not result in the formation of a protein network. The gelation step, induced by the addition of salt or acid, has to be preceded by an activation step in which the protein molecule denatures and forms soluble protein aggregates. This method offers an unique possibility to study the gelation of globular proteins. In the literature this process is known as cold gelation of globular proteins.

The cold gelation process

The principle of cold gelation has been known for some time (De Wit, 1980). Studies really took off in the late 90's with an emphasis on salt-induced, rather than on acid-induced cold gelation. Cold gelation has been reported for preheated solutions of β-lactoglobulin, whey protein concentrates, and whey protein isolates (Vreeker et al., 1992; Barbut and Foegeding, 1993; Sato et al., 1995; Barbut, 1995a/b; Roff and Foegeding, 1996; Hongsprabhas and Barbut, 1996, 1997a/b/c/d; Elofsson et al., 1997; Ju and Kilara, 1998a/b, Kinekawa et al., 1998; Hongsprabhas et al., 1999), for ovalbumin (Kitabatake et al., 1987 and 1988), and for soy protein (Soeda, 1996, 1997).

Compared to heat-induced gelation, the unique feature of the process of cold gelation is that the (heat-induced) activation step of the proteins is uncoupled from the subsequent steps in the gelation process. **Figure 2** schematically depicts the cold gelation process, in which two separate steps can be distinguished. This contrasts with the heat-induced gelation, where the processes of unfolding, aggregation, and gelation are intertwined.

In the first step of the cold gelation process a stable dispersion of protein aggregates is obtained after heating of a solution of native proteins at a pH far from the pI, at low ionic strength (no salts added) and at such a protein concentration that no gel is formed (**Figure 3**). After cooling, a stable dispersion of aggregates is obtained. In the second step, gelation can be induced (marked in **Figure 3** by a non-zero gel hardness) at ambient temperature by changing the solvent quality, for example by adding salt or by lowering the pH.

Heat-induced gelation
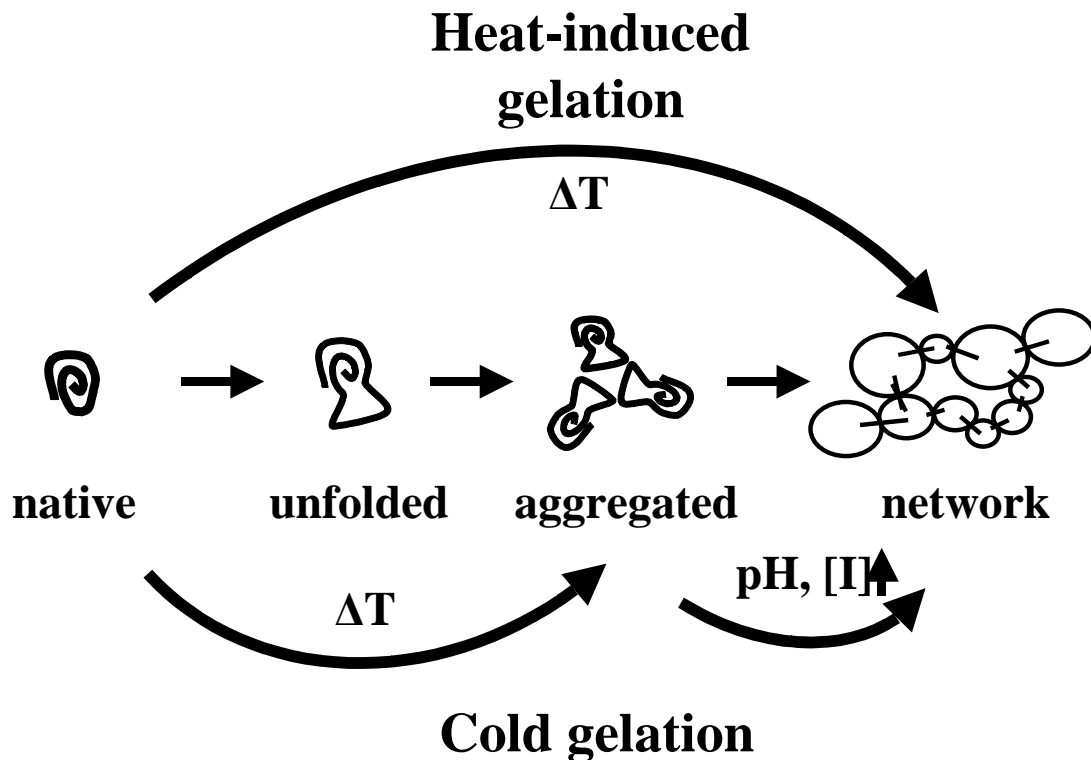
Figure 2: Conversion of native globular protein into a protein network according to the heat-induced or cold gelation procedure. In the heat-induced process, the unfolding (exposure of hydrophobic parts), aggregation and formation of a network are intertwined. In the cold gelation process, the formation of aggregates and the formation of a protein network are clearly separated in time (Alting et al., 2003).

Most studies on the cold gelation process have reported on the application of WPI as cold-gelling ingredient. As reviewed by Bryant and McClements (1998) salt was mainly used to induce gelation. The fact that electrostatic interactions played a dominant role in determining aggregation of heat-denatured protein particles in these type of cold-set gels suggests that also pH should have a large effect.

In the salt-induced type of cold-set gels a fine-stranded transparent gel can be formed at room temperature by adding relatively small amounts of salt. Turbid, particulate gels are formed after addition of relatively large amounts of salt (Barbut & Foegeding, 1993; McClements & Keogh, 1995; Barbut, 1995b). A treatment at $70^{\circ}C$ was required to achieve sufficient structural changes to make the proteins susceptible to Ca^{2+}-induced gelation. Higher activation temperatures and/or longer heating time resulted in higher gel strength as measured by gel penetration experiments (Barbut and Foegeding, 1993). In addition to gel opacity, the calcium concentration affected the water-holding capacity and the gel strength (Hongsprabhas and Barbut, 1996 and 1997a/b). The gel hardness increased with the initial protein concentration at heating, even if the protein concentration in the final gels was made the same (Ju and Kilara, 1998). With the aid of the thiol-blocker *N*-ethylmaleimide, Hongsprabhas and Barbut (1997d) demonstrated that disulfide bonds were mainly involved in the polymerization step prior to gelation and assisted in maintaining the network structure.
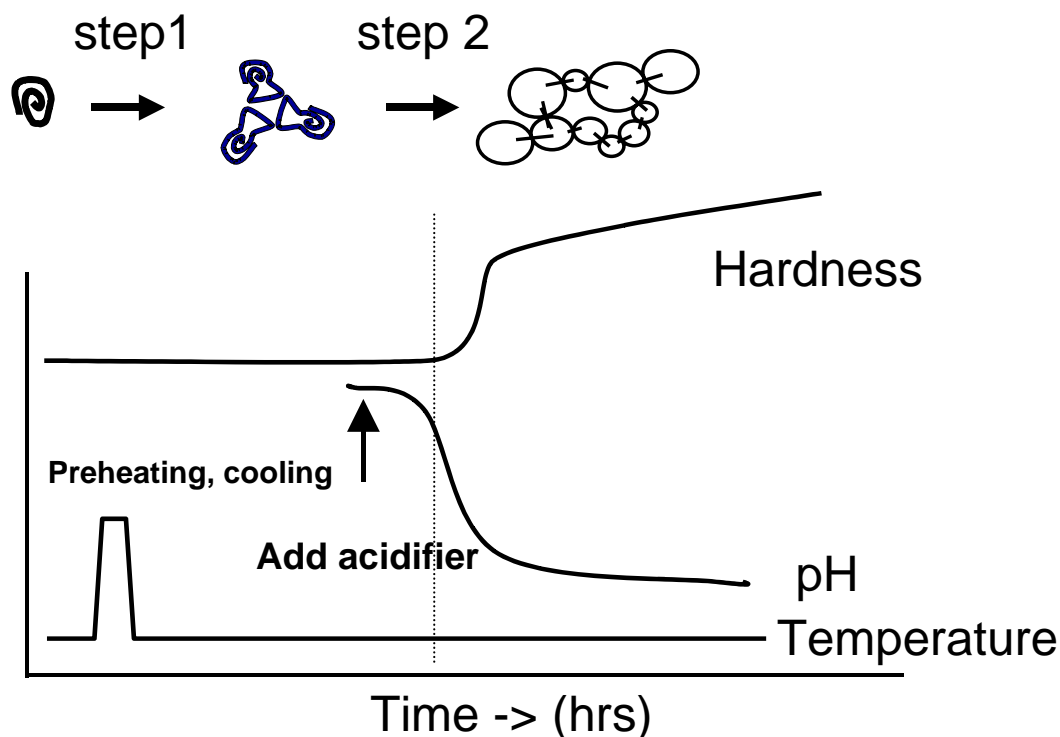
Figure 3: The two-step procedure of acid-induced cold gelation. First, a solution of native protein is heated at a pH far from the pI of the protein and with no salts added. After cooling, a "stable" solution of protein aggregates is obtained. Addition of an acidifier (or base) will decrease (or raise) the pH and gelation is induced at ambient temperature (Alting et al., 2002).

Network formation was governed by the $CaCl_2$ concentration. Variation of the level of $CaCl_2$ resulted in different gelation mechanisms. Electrostatic forces were shown to be more important than covalent bonds in controlling the way gels were formed (Hongsprabhas et al., 1999). To conclude, at the start of this research project little was known about the acid-induced type of cold-set gels compared to the salt-induced type. It was expected that also the structure and properties of this type of cold-set gels will, in addition to the type of protein, and the gelation kinetics, depend on both the amount and properties of the protein aggregates.

Industrial relevance

Application of the cold-gelling method enables the completion of the protein denaturation process before inducing gelation, which allows a more efficient use of protein as building block of a protein network. In addition, in this process, it is possible to determine the properties of the aggregates after heating, but before inducing gelation, and thereby control final gel properties. In a dried form the aggregates can be applied as thickening or gelling agent (Thomsen, 1994). As a processing method, cold gelation finds applications in for example the preparation of heat-sensitive products with a delicate flavor and texture (Barbut, 1995a; Britten and Giroux, 2001), in the production of encapsulates (Beaulieu et al., 2002), or to increase efficiency of iron uptake (Remondetto et al., 2002). Cold-gelling ingredients have a significant potential for new applications, since they do not necessarily need a heat treatment to become functional.

Cysteine-containing globular food proteins

Proteins from different sources, such as plants, eggs, meat, and milk, are used as food ingredients for their structuring properties such as thickening and gelling. The protein preparations

used in this thesis were a commercial whey protein isolate (WPI), β-lactoglobulin (β-lg), and ovalbumin, all water-soluble cysteine-containing globular food proteins. WPI is a mixture of proteins that is widely applied in industrial applications. Therefore it was used throughout this thesis. In addition purified protein fractions of β-lg and ovalbumin were used.

Whey protein isolate (WPI)

Nowadays, whey is a valuable source of high-quality proteins and no longer considered a waste-product of the cheese making process (Smithers et al., 1996). Progress in industrial fractionation and protein isolation techniques has increased the production of whey protein preparations with improved functional and biological properties (Timmer and van der Horst, 1997; De Wit, 2001). In the literature, properties and applications of the whey proteins of bovine milk have been extensively reviewed (McKenzie, 1971; Eigel et al., 1984; Walstra and Jenness, 1984; Mulvihill and Donovan, 1987; Fox, 1989; Kinsella and Whitehead, 1989; Walstra et al, 1999; Foegeding et al., 2002). Here, the properties of the main proteins present in WPI preparations, namely β-lg and α-lactalbumin (α-lac), are briefly reviewed. Especially β-lg, the most abundant protein in WPI (>75%), determines to a large extent the behavior of WPI during heat treatment (Verheul, 1998). In addition to the two major proteins, WPI contains bovine serum albumin (BSA) and immunoglobulins. Since the different proteins may have different denaturation kinetics, the use of protein mixtures instead of pure protein fractions may have an impact on the final properties of the gels when heat-set gels are prepared. However, here cold gelation was used, in which aggregation and gelation take place sequentially, which makes it possible to study the direct relation between aggregate properties and final gel properties. Also in practice complications that arise from the use of protein mixtures are less troublesome since these mainly influence the primary denaturation and aggregation kinetics (Dalgleish et al., 1997), but not the secondary gelation.

β-Lactoglobulin

β-Lg belongs to the lipocalin family of proteins, which is folded as an eight-stranded anti-parallel β-barrel that forms around a central cavity, the calyx. Like most lipocalins, β-lg can bind small hydrophobic molecules within this central cavity. The biological function is as yet unknown, although a role as retinol-transport protein is suggested for bovine β-lg, since it resists peptic hydrolysis during gastric passage. However, this resistance against peptic hydrolysis is not observed for porcine β-lg (Burova et al., 2002). This difference in susceptibility to hydrolysis finds its origin in differences in the tertiary structure of the porcine and the bovine protein (Ugolini et al., 2001; Hoedemaeker et al., 2002). The amino acid sequence of bovine β-lg consists of 162 amino acid residues resulting in a molecular mass of 18.3 kDa and a pI of 5.1. At neutral pH and at ambient temperature bovine β-lg occurs as a dimer, but it dissociates into monomers at higher temperatures or at extreme pH values. Commercially available whey protein preparations contain a mixture of the A and B variants of β-lg (Visser & Slangen, 1994) that both have one cysteine residue and two cystines. One buried in the tertiary structure and another positioned at the outer surface in a more mobile region of the molecule. Especially the thiol group of the cysteine residue (amino acid residue 121) which is exposed during heat-induced unfolding, is important for the aggregation behavior of β-lg (Roefs & De Kruif, 1994). Since the genetic variants do not differ in the number and positions of the cysteine and cystine residues, these are not taken into account in this study. By analogy with the free radical addition polymerization reaction, the exposed thiol-group is able to initiate intermolecular thiol/disulfide interchange reactions and to form intermolecular disulfide bonds by thiol-oxidation reactions (Roefs & De Kruif, 1994). In good agreement with this model, β-lg treated with *N*-ethylmaleimide (NEM), which blocks the thiol group of the cysteine residue, gives no polymerization after heating (Hoffmann & Van Mil, 1997). Kitabatake et al. (2001) reported that the conformation of the NEM-treated protein molecule after heating was the same as before heating.

Further evidence of the importance of the free thiol group is the fact that no gelation occurs for porcine β-lg, which lacks the cysteine residue present in the bovine variant (Gallagher et al., 1996). Moreover Burova et al. (2002) reported that the heat-denaturation of porcine β-lg is reversible due to the absence of the cysteine residue.

α-Lactalbumin

α-Lac is an acidic, monomeric, calcium-binding metallo-protein. The sequence and the tertiary folding of α-lac is homologous to that of proteins of the lysozyme family. In spite of that, its cell-lytic activity is negligible compared to that of egg lysozyme. In its biological function α-lac is involved in the synthesis of lactose by regulation of the activity of the enzyme galactosyltransferase. It promotes the binding of glucose to galactosyltransferase, which enhances the transfer of galactose from uridine diphosphogalactose to glucose (Permyakov & Berliner, 2000). The protein consists of 123 amino acids, including cystines, and has a molecular mass of 14.2 kDa. Three genetic variants, A, B, and C, are known. In the milk of western breeds only the B variant is present. The pI of α-lac is 4.2-4.5 (Verheul, 1998). The binding of calcium to α-lac causes pronounced changes in its tertiary structure. Removal of calcium reduces the heat stability of the protein. Several authors have concluded that α-lac on its own does not form aggregates upon heating (Dalgleish et al., 1997; Rojas et al., 1997). The presence of thiol-containing proteins, such as β-lg or BSA are a prerequisite to induce aggregation, which is in line with the free radical addition polymerization model (Roefs & De Kruif, 1994).

Ovalbumin

The avian egg white contains 13 well-characterized proteins, of which ovalbumin, ovotransferrin (conalbumin), and ovomucoid account for 77% of the total protein, and among which ovalbumin is the predominant protein (60-65%) (Powrie & Nakai, 1985). As for β-lg, the biological function of ovalbumin is still unknown (Huntington & Stein, 2001). Ovalbumin belongs to the protein super-family of serine protease inhibitors (serpins). The functional activity of serpins as protease inhibitors depends on their unique ability to undergo a dramatic conformational change on interaction with an attacking protease, although ovalbumin lacks this protease inhibitory activity (Hunt & Dayhof, 1980). The complete amino acid sequence of 386 residues is known, containing an acetylated N-terminus. The molecular mass is 45 kDa. Three types of ovalbumin, varying in the degree of phosphorylation, are known, A1, A2, and A3, respectively, with different pI's, 4.75, 4.89, and 4.94, respectively. The available ovalbumin preparations are often a mixture of these three protein variants. As for the whey proteins, in this thesis neither the two genetic variants, nor the differences in degree of phosphorylation were taken into account. Ovalbumin contains four cysteine residues and one cystine (in Doi & Kitabatake, 1997).

Purpose of the study

Texture plays a pivotal role in the sensory properties of food products. It is one of the major criteria which consumers use to judge the quality and freshness of foods. This study was part of a project that was aimed at achieving a better control of the structural properties and stability that result from aggregation, gelation and gelation-arrested phase separation of food biopolymers, in a way that is relevant for the Dutch food industry.

The purpose of the research described in this thesis was to investigate the molecular mechanism of the acid-induced cold gelation process in order to better control and exploit the resulting gel structures in food applications. The unique advantage of this method, the possibility to determine and to manipulate aggregate properties before inducing gelation, was exploited to identify the key factors that regulate this process. The contributions of noncovalent (physical) and covalent (chemical) interactions to the kinetics of gelation and to final properties of acid-induced

cold-set protein gels were systematically studied. Within this systematical study, the role of thiol groups in the case of cysteine-containing globular proteins was highlighted. The results will enable us to link mesoscopic properties of aggregates to macroscopic characteristics of food gels in general, and of cold-set gels in particular, allowing a better use of this alternative method in food applications.

Outline of the thesis

In this chapter a brief overview has been given of the gelation of globular proteins in general and of the cold gelation of whey proteins in particular.

Chapter 2 describes the initial work on the role of the additional formation of disulfide bonds in the second step of the cold gelation process of whey proteins. In this study advantage was taken of the possibility to modify aggregates before gelation was induced. Thiol groups, present on the aggregates produced in the first step of the process, were modified by the use of different thiol-blocking agents. In this way it was demonstrated that during acidification, initially an acid-induced protein network was formed by physical interactions and that subsequently this network was stabilized by the additional formation of disulfide bonds, which contributed to an increased gel hardness.

In chapter 3, the net charge of β-lg and whey protein aggregates was modified, either by succinylation of the primary amino groups or by methylation of the carboxylic acid groups, in order to establish the contribution of electrostatic interactions in the cold gelation process. This study clearly showed that reduction of the electrostatic interactions was the driving force in acid-induced gelation and that the formation of disulfide bonds during the gel state only occurred above pH values of 3.5.

The previously reported effect of the aggregate size on the mechanical properties of cold-set WPI-gels was investigated in the work described in chapter 4 by the use of thiol-blocked aggregates. Aggregates varying in size were produced by varying the protein concentration at heating. From this work we concluded that the number of thiol groups rather than the size of the aggregates determined the hardness of cold-set WPI gels.

Chapter 5 describes studies on the interactions between reactive aggregates, the structure and rheology of cold-set gels as a function of the protein concentration. From the results of this chapter it was suggested that acid-induced cold gelation probably starts off as a random aggregation process, but is taken over by a phase separation mechanism at larger length scales (> 100 nm). In addition, indications were found for disulfide cross-link-dependent structural rearrangements at smaller length scales (< 100 nm).

In chapter 6 the cold gelation of ovalbumin was compared with that of WPI. Protein-specific and more general mechanisms in the cold gelation process could be distinguished. In spite of the presence of reactive thiol groups on the surface of ovalbumin aggregates, no formation of disulfide bonds during the acidification step was observed.

Applications of the knowledge obtained in this thesis are presented in chapters 7 and 8. Chapter 7 describes that by means of microbial acidification mechanical properties could be set by variation of the acidification curve or rate. Mechanical properties of cold-set gels of both ovalbumin and WPI could be controlled by the final pH and by the rate of acidification. Both electrostatic interactions and formation of disulfide bonds were shown to depend on these processing conditions. Based on these results a patent application was filed.

Chapter 8 reports experiments on the additional formation of disulfide bonds between whey protein aggregates and whey-protein-covered casein micelles in acid-induced gels of heated milk and their effect on the texture. Addition of the thiol-blocking agent N-ethylmaleimide prevented the formation of disulfide-linked structures in acidified heated milk. The mechanical properties of acidified heated milk were shown to be the result of the contribution of denatured whey proteins to the protein network as such and the additional formation of disulfide bonds. As in cold-set WPI

gels, these thiol group-disulfide bond exchange reactions took place at ambient temperature and under acidic conditions. Therefore, the disulfide cross-linking is highly relevant for textural properties of acid-milk products, like yoghurt.

In chapter 9 the results are summarized and concluding remarks are presented.

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Chapter 2

Formation of disulfide bonds in acid-induced gels of preheated whey protein isolate

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

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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

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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, 19000g, 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 such 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).

Disulfide Bond Formation during Cold Gelation

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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

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.

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.

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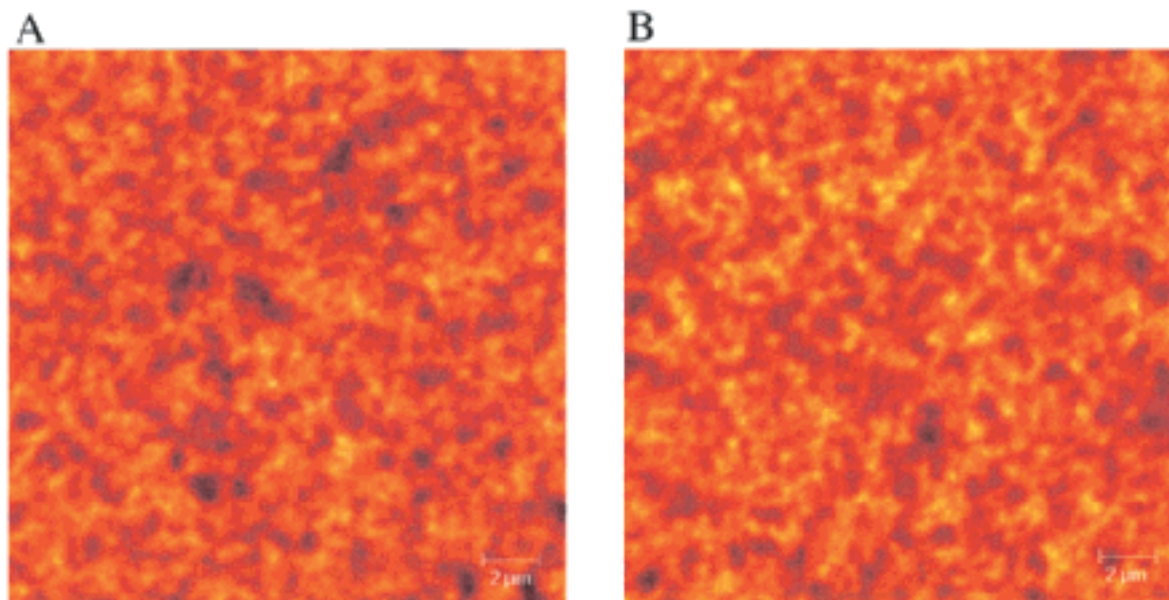
Figure 1. CSLM images of 2% WPI gels (A), and gels of WPI after a treatment with NEM (B). A fluorescent dye, Rhodamine B, was applied to the gels. The dye binds noncovalently to the protein network. Light areas in the images reflect higher dye concentrations and therefore represent the protein network.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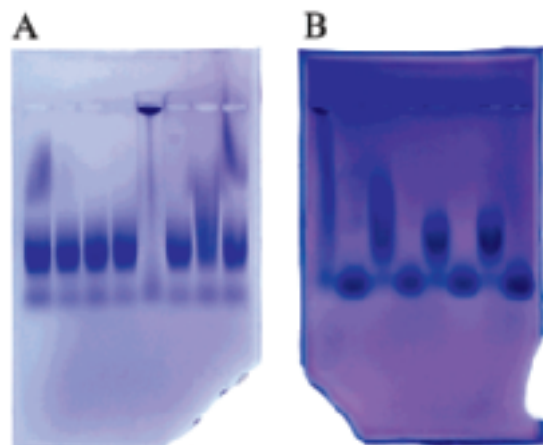

Figure 2. (A) SDS−agarose gel electrophoresis of WPI aggregates: effect of thiol-blocking agents on the molecular weight of the aggregates before and after gelation and resolubilization. The four lanes on the left-hand side of the gel contain the different aggregates before gelation: without thiol-blocking 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

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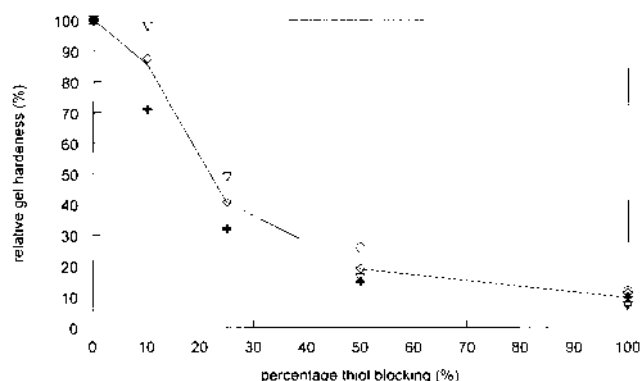
Figure 3. Effect of the amount of thiol-blocking (NEM) on the relative gel hardness of acid-induced gels. Results of the different experiments are represented as different symbols. The solid line connects the average values of the experiments.

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 pre-heating 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-

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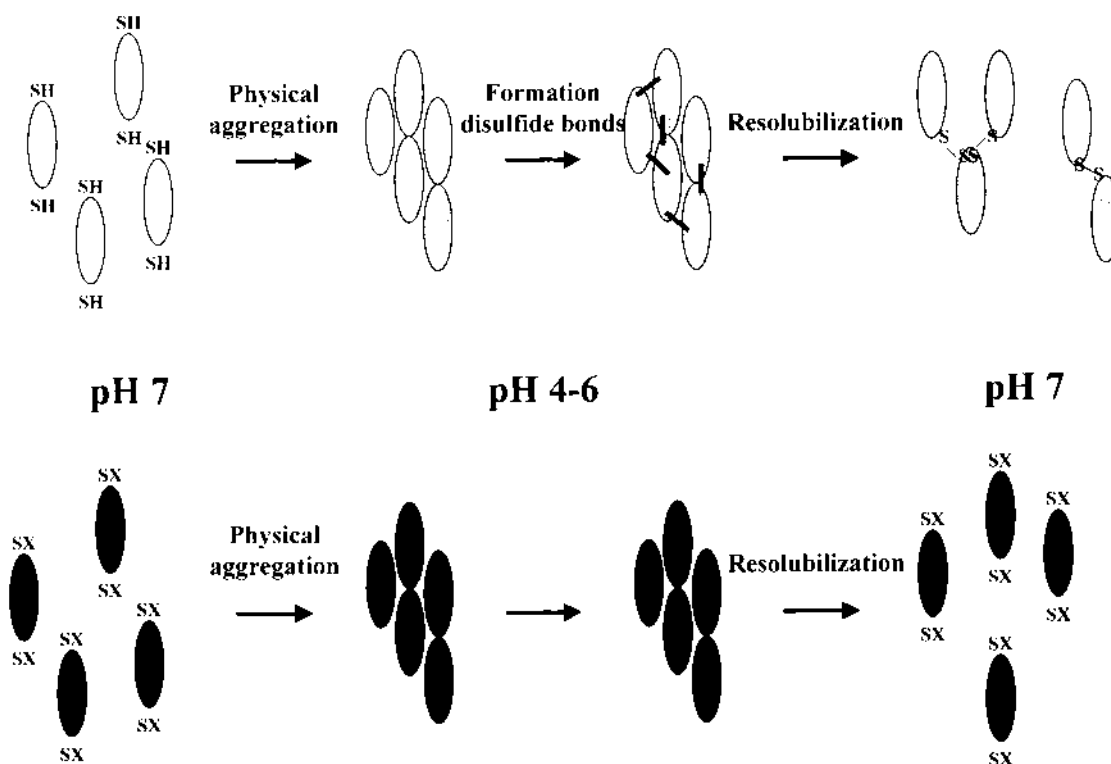
Figure 4. Model for the formation of intermolecular disulfide bridges and their role during the acid-induced cold gelation of heat-treated whey proteins. The oval shapes represent the WPI aggregates after heating (size ∼80 nm). The upper part describes the gelation process of unblocked aggregates. In 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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Chapter 3

Physical and chemical interactions in cold gelation of food proteins

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Physical and Chemical Interactions in Cold Gelation of Food Proteins

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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*).

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

	hydrodynamic diameter (nm)[b]	
sample	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
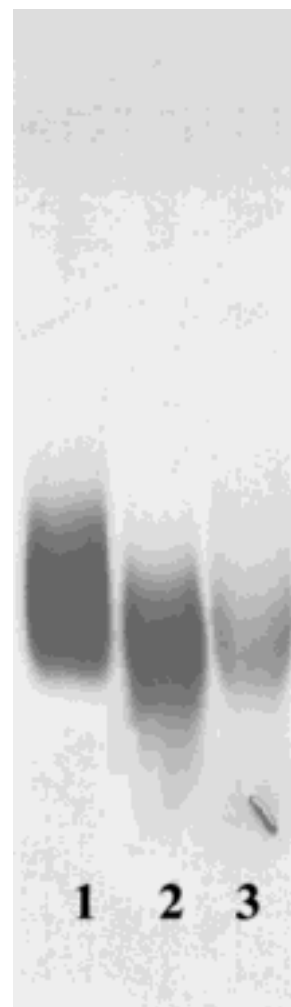
Figure 1. Agarose gel electrophoresis (in the absence of SDS) of nonmodified and succinylated WPI aggregates. Proteins were stained according to the standard Coomassie brilliant blue staining method: lane 1, nonmodified WPI-aggregates; lane 2, 56% succinylated WPI-aggregates; lane 3, 93% succinylated WPI aggregates.
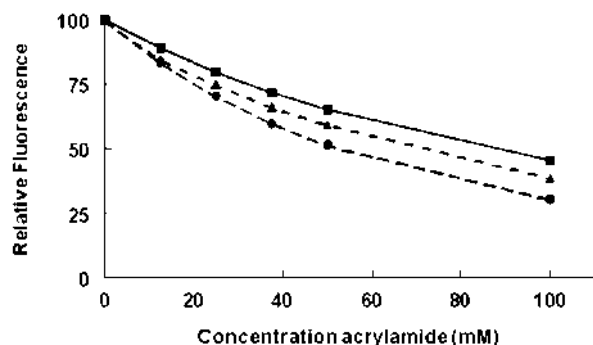
solution was calculated from a cumulant fit of the intensity autocorrelation function. Before analysis, samples were filtered through a low-protein binding membrane (5 μm; Millex-SV, Millipore Corporation, Bedford, MA).

Table 2. Characterization of β-Lg and WPI Aggregates

	concentration of amino groups		degree of modification (%)	calculated pI-shift[c]	shift in pH/turbidity curve[d]	concentration of thiol groups (mM)[e]
sample	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.

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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
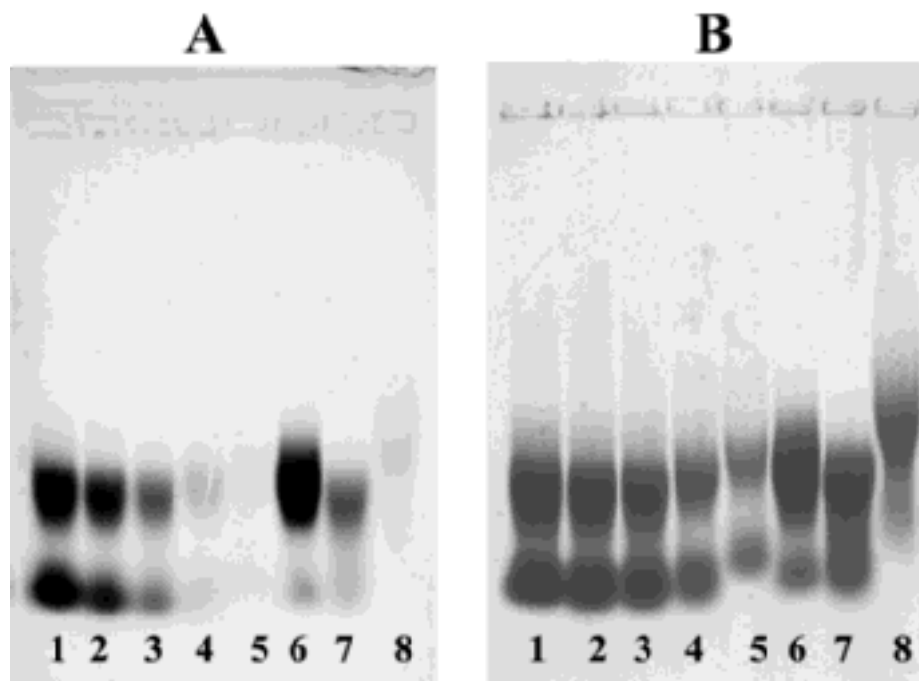
Figure 3. SDS-agarose gel electrophoresis of nonmodified and succinylated β-Lg and WPI aggregates. Proteins were stained according to the standard Coomassie brilliant blue staining method (gel A) and according to an adapted method from Hegenauer et al. (*25*) (gel B). β-Lg aggregates: lane 1, nonmodified; 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.

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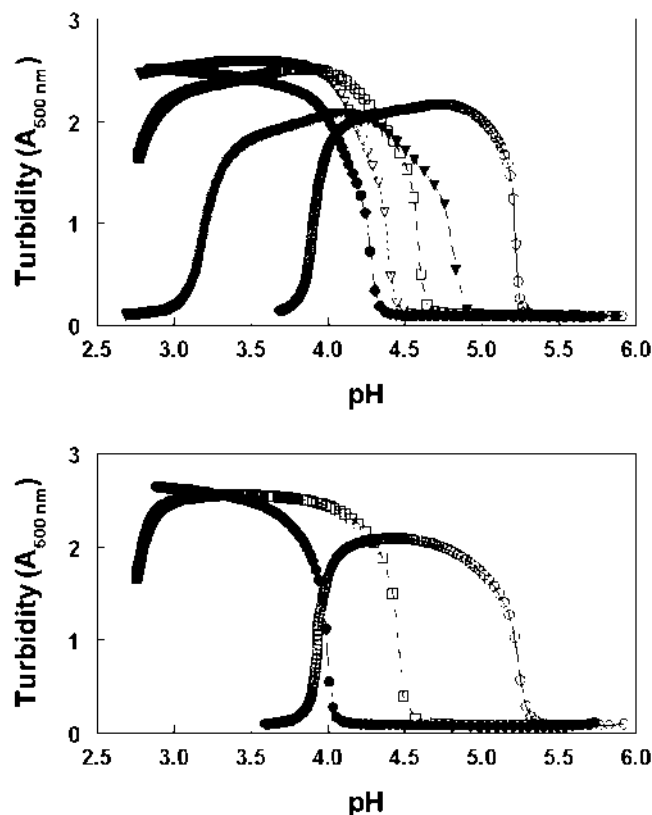
Figure 4. Dependence of the turbidity on the pH for solutions of non-modified and succinylated β-Lg (A) and WPI-aggregates (B). The turbidity was measured in time as the absorbance at 500 nm. Samples were measured in cuvettes with a path length of 2 mm. The pH was monitored simultaneously. Turbidity 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.
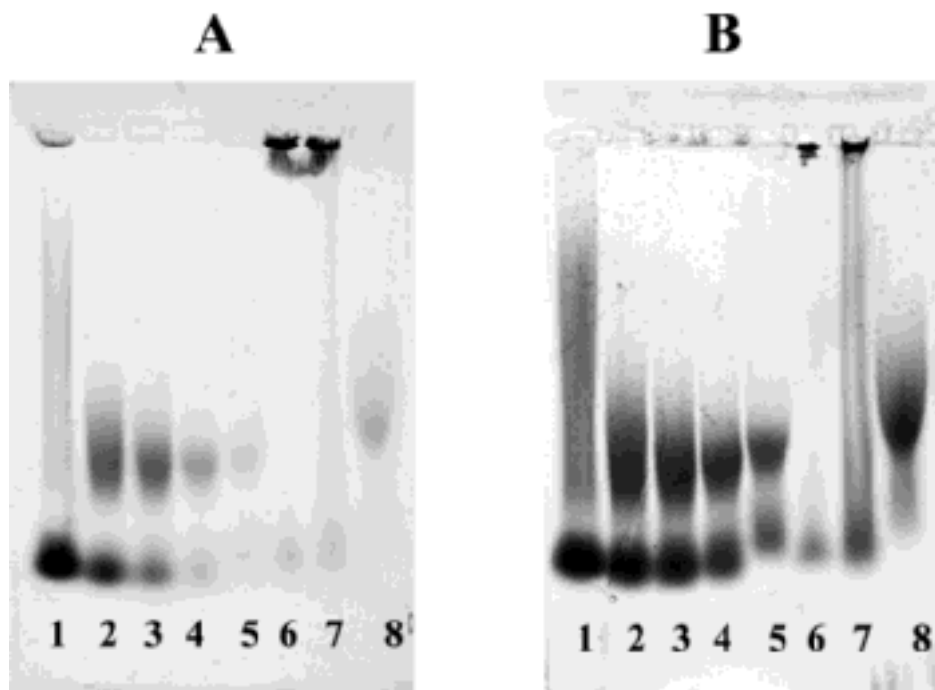
mobility of the aggregates (upper bands) is decreased compared to the mobility of the monomeric protein (lower bands). Although for both the β-Lg and the WPI solutions, the majority of the protein is present as aggregates, the amount of residual monomeric protein after heat treatment for β-Lg is higher 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 elec-trophoretic 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 concentra-tions 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 be-tween 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 ag-gregates (**Figure 4B**). It is clear from **Figure 4** that the aggregation curves for the modified aggregates have shifted

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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.

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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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Chapter 4

Number of thiol groups rather than the size of the aggregates determines the hardness of cold-set whey protein gels

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Number of thiol groups rather than the size of the aggregates determines the hardness of cold set whey protein gels[☆]

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Abstract

Variation of protein concentration during heating resulted in the formation of protein aggregates with clearly different structural and chemical characteristics. Heating conditions were chosen such that differences in the degree of aggregation were excluded. Acid induced gelation of dispersions of these aggregates resulted in gels with clearly different hardness. Although gel hardness seemed to correlate with the different structural aggregate features as reported before in literature, the differences in hardness could for the most part be cancelled by blocking of the thiol groups. Application of thiol-blocked protein aggregates enabled us to make a distinction between the effect of structural- and chemical-properties of the aggregates. Formation of larger disulfide cross-linked protein structures paralleled the increase in gel hardness and dominated the effect of structural characteristics on mechanical properties of cold-set gels. In addition, the effect of the presence of native non-aggregated protein on the final gel properties can be excluded, since in our gel-experiments most protein ($>95\%$) participated in the formation of a protein network. Therefore, we can conclude that the hardness of cold set whey protein gels is determined by the number of thiol groups rather than by the size of the aggregates or other structural features.
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Keywords: Cold gelation; Disulphide bonds; Whey proteins; Aggregate size; Thiol-blockers; Whey protein isolate

1. Introduction

Heat-induced aggregation and gelation of whey proteins has been extensively studied and reported and has found application in a wide variety of products. Cold set gelation (Bryant & McClements, 1998) can also be used in a variety of products, such as surimi, mayonnaise and gelatin-like deserts (Barbut, 1995). This method of gelation consists of two steps. First a solution of native (whey) proteins is heated at neutral pH (well above their iso-electric point), at low ionic strength and a protein concentration lower than the gelation concentration. For whey protein isolate (WPI), this latter concentration is reported to be around 12% (w/w) (Hongsprabhas & Barbut, 1997; Otte, Ju, Skriver, & Qvist, 1996). In this first step, unfolding of the native proteins is followed by aggregation into disulphide cross-linked aggregates (Roefs & de Kruif, 1994). Under the described conditions, the proteins have a net surface charge and repulsive forces will prevent random aggregation, resulting in the formation of soluble linear or curved ellipsoid-shaped aggregates (Doi, 1993; Le Bon, 2001). After cooling to room temperature a stable dispersion of aggregates is obtained. In the second step, gelation is induced by changing the solvent quality. A fine-stranded, transparent gel can be formed at room temperature by adding relatively small amounts of salt. Turbid, particulate gels are formed after addition of relatively large amounts of salt or after acidification towards the iso-electric point (Alting, De Jongh, Visschers, & Simons, 2002; Alting,

Hamer, De Kruif, & Visschers, 2000; Barbut, 1995; Barbut & Foegeding, 1993; McClements & Keogh, 1995). Usually, acid-induced cold-set gels are mechanically stronger than salt-induced cold-set gels of the same protein concentration (Ju & Kilara, 1998)

The structure and properties of cold-set gels of whey proteins will, in addition to the gelation kinetics and type of induction, depend on both the number and properties of aggregates. Contrary to heat-induced gelation, in the process of acid-induced cold gelation it is possible to determine and manipulate aggregate properties before inducing gelation. In a previous study, we modified the WPI-aggregates by blocking the reactive thiol groups on the surface of the aggregates (Alting et al., 2000). In this way, the additional formation of disulphide bonds during the gelation step of aggregates without thiol-blocking treatment was clearly demonstrated, and explained quantitatively the increase in gel hardness. The importance of the net-charge of the aggregates in cold-set-gels was demonstrated by succinylation of the primary amino groups and by methylation of carboxylic acid groups, showing that the gelation point is determined by the iso-electric point of the protein used (Alting et al., 2002).

In addition to the amount of thiol groups and the net-charge, the size of the aggregates is expected to be important for determining gel properties. Ju and Kilara (1998) studied the effect of the aggregate properties on gelation and concluded that the hardness of cold-set gels increased with increasing aggregate size and degree of aggregation, i.e. the fraction of denatured protein relative to the total amount of protein. The effect of degree of aggregation was earlier reported. Barbut and Foegeding (1993) observed that the hardness of cold-set gels depends on the temperature and heating time (relative degree of aggregation) during the preparation of aggregates. Also more recently, Mleko (1999) suggested that the gel properties of heat-set WPI-gels prepared in a two-stage heating process were significantly influenced by differences in aggregate size.

For β-lactoglobulin, the main constituent of WPI, it is reported that the heat-induced aggregation depends on the temperature-time combination, but also on the pH, type and concentration of salt and the protein concentration (Hoffman, 1997). The average size of the protein aggregates can be varied by changing the protein concentration at heating. The aggregates formed are mainly held together by intermolecular disulphide bonds (Hoffmann & Van Mil, 1997; Roefs & De Kruif, 1994). The WPI-aggregates are considered to be co-aggregates of the various whey proteins, α-lactalbumine, β-lactoglobulin, bovine serum albumin and serum globulins (Ju, Otte, Zakora, & Qvist, 1997). Also the average aggregate size of the WPI-aggregates depends on the initial protein concentration at heating (Ju & Kilara, 1998), pH and calcium concentration at heating (Britten & Giroux, 2001).

The purpose of this study was to ascertain the effect of the aggregate size on the mechanical properties of cold-set

WPI-gels. By the use of thiol-blocked-aggregates, a distinction between the effect of the aggregate size and the contribution of thiol groups on the hardness of cold-set gels of WPI becomes possible. Varying protein concentration while maintaining constant heating time and temperature profiles may lead to incomplete aggregation. As the fraction of native proteins will not participate in the building of an acid-induced protein network, an effect of the degree of aggregation on mechanical gel properties is expected. Therefore, in this study, solutions of WPI-aggregates with in excess of 95% of the native proteins participating in aggregate formation were applied.

2. Material and methods

2.1. Reagents and chemicals

Glucono-δ-lactone (GDL), 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), Sodium dodecylsulphate (SDS) and *N*-ethylmaleimide (NEM) were obtained from Sigma Chemicals (St Louis, MO. USA). Electrophoresis grade agarose was obtained from Life Technologies (Paisley, Scotland). Phastgel blue R tablets were from Pharmacia Biotech (Uppsala, Sweden). The WPI Bipro was a kind gift from Davisco Foods International Inc. (Le Sueur, MN, USA).

2.2. Preparation of soluble WPI-aggregates

WPI was dissolved in double distilled water to a concentration of 3, 4.5, 6, 7.5 and 9% (w/w) and stirred for at least 2 h (Verheul & Roefs, 1998). The WPI-solutions were centrifuged (30 min 19,000g 20 °C) and the supernatant was filtered (0.22 μm; Millex-SV, Millipore Corporation, Bedford, Mass.). Reactive WPI-aggregates were prepared by heating the WPI-solutions in a water bath for, respectively 2, 4, 7, 21 or 24 h at 68.5 °C. The solutions were cooled with running tap water or in ice-water. The amount of native proteins after heat treatment was determined with a standard assay involving acid precipitation and gel-permeation chromatography (Hoffmann, Roefs, Verheul, Van Mil, & De Kruif, 1996). The dispersions of WPI-aggregates were diluted with filtered (0.22 μm; Millex-GV, Millipore Corporation, Bedford, Mass.) double distilled water to a protein concentration of 2% (w/w) and stored at 4 °C. Sodium azide (0.02% (w/w) final concentration) was used as a preservative. The headspaces were filled with nitrogen to prevent oxidation of thiol groups during storage.

2.3. Blocking of the free thiol-groups

Soluble reactive aggregates (2% (w/w), protein concentration after dilution) were treated with NEM (1 mM). Previous work has shown that 0.5 mM NEM was sufficient to block the accessible thiol groups (Alting et al., 2000).

After 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.

2.4. Accessibility of the thiol-groups

Accessible thiol groups before and after treatment with the sulphydryl reagent, NEM were determined using Ellman's reagent (Ellman, 1959) essentially as described by Hoffmann and Van Mil (1997) with the exception that a Bis-Tris/HCl buffer (pH 7) was used instead of a Tris/HCl buffer. The assay was performed in the absence of detergents like urea or SDS, since under these conditions only the thiol-groups of interest, those at the surface of the aggregates, were determined. Experiments were performed in duplicate and the number of thiol groups was calculated using a molar extinction coefficient for 2-nitro-5-mercaptobenzoic acid of 13,600 M^{-1} cm^{-1}.

2.5. Voluminosity and intrinsic viscocity

The voluminosity (Φ) of WPI-aggregates in very dilute solutions of WPI-aggregates was determined according to Tuinier, Dhont, and De Kruif (2000), using Einstein's expression $\eta_r = 1 + 2.5\Phi$. In this expression η_r stands for the relative viscosity η/η_s, where η is the solution viscosity and η_s the viscosity of the continuous phase. The concentration dependence viscosity of dilute solutions of WPI-aggregates (0–6 g/l) (heated at 68.5 °C at different protein concentrations and for different time intervals) were measured with an Ubbelohde capillary viscometer at 20 °C. The voluminosity was determined from the initial slope of the concentration dependence of η_r using Einstein's expression (Φ = slope × proteinconcentration/2.5).

The intrinsic viscosity was determined according to Vardhanabhuti and Foegeding (1999) on the basis of the Huggins equation: $\eta_{sp}/c = [\eta] + k[\eta]^2 c$. Extrapolation of the plot of η_{sp}/c versus c to zero protein concentration gives the intrinsic viscosity. In this equation c stands for the concentration of protein (g/ml). The specific viscosity, η_{sp}, was calculated from $\eta_{sp} = (t - t_0)/t_0$, where t_0 is the efflux of water and t the efflux of protein solution in the viscosity measurement using an Ubbelohde capillary viscometer (20 °C).

The calculations of both the intrinsic viscosity and the voluminosity are based on at least 10 measurements of the efflux times of the different protein solutions and dilutions thereof.

2.6. Cryo-transmission electron microscopy

All samples (dispersions of protein aggregates) obtained a 10 times dilution using millipore-filtered (0.2 μm pore size) distilled water. Wholey carbon grids obtained from Quantifoil (R 2\1, 400 mesh Cu grids) and Agar Scientific (S147-3, 300 mesh Cu grids), were used. Prior to sample preparation the grids were subjected to a glow-discharge (ion-bombardment) treatment to make them hydrophyllic. Grids were handled using a fine-tipped pair of tweezers, containing a thermal insulating coating to prevent heat transfer from the hand through the freezers to the specimen. To create a thin film a grid was dipped into the diluted protein dispersion, blotted with filter paper (Schleicher and Schuel, zwart-band 589, circular 90 mm in diameter) to remove excess sample, directly followed by plunging into liquid propane (liquid nitrogen cooled, −187 °C) using the Reichert-Jung KF80 plunging device. All handling steps below were executed under liquid or cooled gaseous nitrogen to prevent any heating up of or frost formation on the specimen. The access of liquid propane was removed by blotting with a pre-cooled filter paper, followed by transfer of the specimen into a transfer holder filled with liquid nitrogen, followed by transfer to the location of the transmission electron microscope (TEM). The specimen was inserted in the pre-cooled (liquid nitrogen) Oxford cryo-transfer holder using a loading station and inserted in the TEM for observation. Observations were done in low-dose mode at 120 kV accelerating voltage and image recording done by the Gatan 694 slow scan CCD camera.

2.7. Preparation and solubilization of acid-induced gels

GDL was added to the 2% (w/w) WPI-solutions to induce cold gelation. Typically, such an amount of GDL (0.14%) was added that at ambient temperature the pH of the solution was gradually lowered from pH 7.2 to a pH of around 5 (after approximately 24 h). This acidification induced gelation of the WPI-solution. Gel samples were mixed with a buffer containing SDS (see Section 2.8) to solubilize the gels.

2.8. Agarose gel electrophoresis

SDS–agarose continuous gel electrophoresis (0.4% agarose) was performed as described previously (Alting et al., 2000) to determine the differences in molecular weight of the different treated WPI-aggregates. In short, aggregates (in solution or gel) were mixed with three parts of 20 mM Bis–Tris buffer (pH 7.0) 5% SDS and were held at ambient temperature while constantly stirred. After overnight incubation no gel particles could be observed with a standard microscope (at 400 × magnification). Gels were stained by the use of Phastgel blue R (Pharmacia Biotech, Uppsala, Sweden).

2.9. Dynamic light scattering experiments

Dynamic light scattering (DLS) experiments were performed as outlined by Verheul, Roefs, and De Kruif, (1998). The apparent diameter of the aggregates in solution was calculated from a standard cumulant fit of the intensity autocorrelation function, assuming that the aggregates have

a roughly spherical shape (Hoffmann et al., 1996). Before analysis, samples were filtered through a low-protein binding membrane (5 μm; Millex-SV, Millipore Corporation, Bedford, MA, USA). Experiments were at least performed in duplicate with an experimental error lower than 5%.

2.10. Gel hardness

Gel hardness was determined by a texture analyzer (type TA-XT2, Stable Micro Systems ltd, Godalming, England). GDL (0.14%, w/w) was added to 100 g of a 2% dispersion 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 ($45 \times 1.5 \times 2$ mm^3) of stainless steel arranged in a double cross. A force-time curve was obtained at a crosshead speed of 0.3 mm/s 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). All experiments were at least performed in duplicate and the experimental error was lower than 10%.

2.11. 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 (Leica Microsystems, Rijswijk, The Netherlands). The protein gels were stained by applying 2 μl of an aqueous solution of 0.05% Rhodamine B. The dye binds non-covalently to the protein network. The 568 nm laser line was used for excitation inducing a fluorescent emission of Rhodamine B, detected between 600 and 700 nm. The program Qwin (Image analysis package of Leica Microsystems) was applied to quantitatively analyze the CSLM-images in order to determine the relative pore area.

3. Results and discussion

3.1. Preparation of soluble aggregates

WPI solutions which differ in protein concentration (3–9%) were heated for different time intervals at 68.5 °C to evaluate the effect of the initial protein concentration on the size of the aggregates. Fig. 1 shows that upon heating the concentration of native whey proteins decreased and aggregates of disulfide cross-linked whey proteins were formed, which is in agreement with the findings of Hoffmann, 1997. Different heating times had to be applied to obtain dispersions of aggregates with in excess of 95% of the native proteins participating in aggregate formation. Solutions with lower protein concentrations were heated longer to reach the same degree of aggregation. To reach
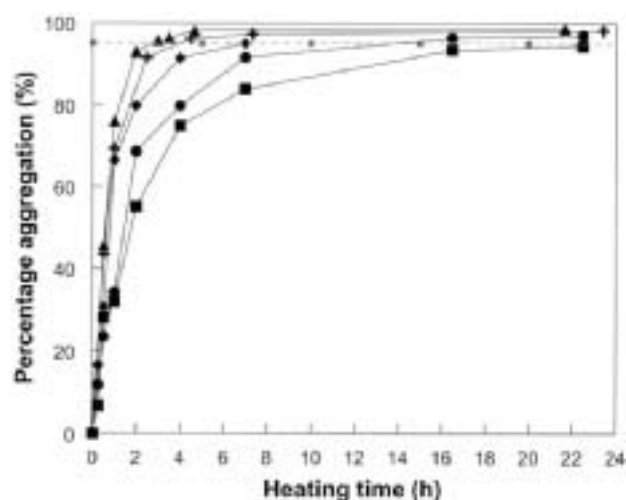

Fig. 1. The protein concentration (w/w) dependence of the denaturation/aggregation of whey protein (WPI) during heating for different time intervals at 68.5 °C. (squares, 3%; circles, 4.5%; diamonds, 6%; crosses, 7.5% and triangles, 9%). The dotted line represents 95% aggregation of protein.

more than 95% aggregation, protein solutions with an initial concentration of 3, 4.5, 6, 7.5 and 9% (w/w) were heated for 24, 21, 7, 4 and 2 h, respectively. It is expected that the fraction of native proteins ($<5\%$) that remained after heating is so low that it will not affect the mechanical properties of the final acid induced cold-set gels.

3.2. Properties of soluble aggregates

In the literature (Britten & Giroux, 2001; Ju & Kilara, 1998) different structural properties of the aggregates (hydrodynamic diameter, intrinsic viscosity) were related to mechanical properties of cold-set gels. Previously, we have demonstrated the importance of the formation of disulphide bonds on the mechanical properties of cold-set gels. Also in this study we expect a significant contribution of the formation of disulfide bridges on gel hardness. Therefore, aggregates were also characterized on their number of thiol groups. In order to determine which of the properties of the aggregates relates to the hardness of acid-induced cold-set gels, aggregates prepared in this study were characterized by both biochemical and physical-chemical analysis techniques.

3.2.1. The number of thiol groups

The thiol groups present on the native whey proteins, especially on β-lactoglobulin are buried in the tertiary structure of the native protein molecule. Denaturation of β-lactoglobulin will make these thiol groups accessible for further reaction (Roefs & De Kruif, 1994). The initial exposure of reactive thiols paralleled the denaturation and aggregation of the proteins (Fig. 2). It was further observed that at longer heating times, the number of available thiol groups decreased. Degassing before heating and heating
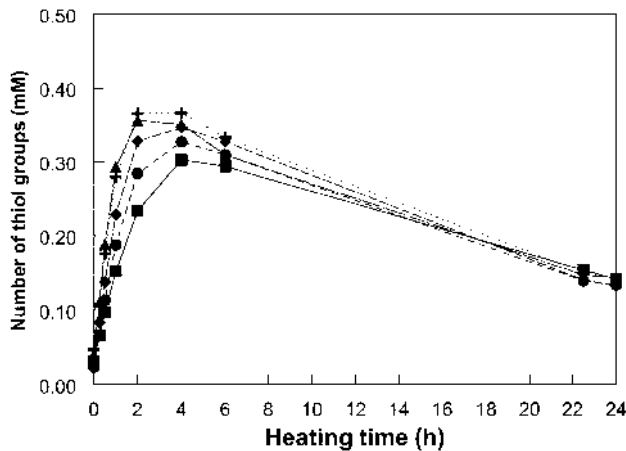
Fig. 2. Liberation and oxidation of thiol groups during the heating of WPI-solution at different protein concentrations (w/w) (squares, 3%; circles, 4.5%; diamonds, 6%; crosses, 7.5% and triangles, 9%).

under nitrogen, did not significantly help to prevent this. Also storage of the solutions of aggregates ($>95\%$ aggregated) under air caused a decrease in the total amount of detectable thiol groups. Storage under nitrogen did prevent further loss of thiol groups. At the point in time that 95% of the protein was aggregated, a lower number of thiol groups was detectable for aggregates prepared at a lower initial protein concentration (Table 1). Note that between an initial protein concentration (at heating) of 4.5 and 6% a relatively large difference in the number of detectable thiol groups is observed. As the aggregates produced by heating at 3 and 4.5% protein were heated for a relatively long period to reach 95% aggregation, the lower number of detectable thiol groups could be explained by oxidation, either by reaction with oxygen or by reaction of two thiol-groups. The oxidation with oxygen is not very likely since heating under nitrogen had hardly any effect. The decrease in thiol group content may also have been

Table 1

The number of detectable thiol groups in solutions of WPI-aggregates of different initial protein concentration at different stages in the heating process

Concentration protein at heating (%)	Number of detectable thiol groups at $>95\%$ aggregation in a 2% solution of protein (mM)[a]
3	0.09
4.5	0.14
6	0.25
7.5	0.30
9	0.32

[a] Determined at pH 7 with the Ellman's assay (Ellman, 1959). The number of thiol groups was determined using $\epsilon(412\ nm) = 13,600\ M^{-1} \times cm^{-1}$ for 2-nitro-5-mercaptobenzoic acid and expressed as the concentration thiol groups (mM) in a 2% (w/w) dispersion of protein aggregates. Measurements were done in duplicate with an experimental error lower than 10%.

caused by degradation reactions, yielding H_2S and dehydroalanine (Nashef et al., 1977; Watanabe & Klostermeyer, 1976).

3.2.2. Hydrodynamic diameter

The effect of the initial protein concentration on the size of the aggregates ($>95\%$ aggregated) was studied by DLS. In agreement with Ju and Kilara (1998) heating at initial protein concentrations from 3 to 9% (w/w) led to an increase of the hydrodynamic diameter yielding aggregates with a size ranging from 29 to 69 nm (Table 2). In contrast to Ju and Kilara (1998), but in line with the observation of Le Bon (2001), who studied the aggregation of β-lactoglobulin, under a critical initial protein concentration aggregates with a diameter of around 30 nm were obtained. Above this critical concentration the diameter increased with increasing protein concentration at heating. These results were explained by the subsequent aggregation of primary aggregates above a critical protein concentration into larger structures and finally into a space-filling protein network. The dependence of size of the aggregates on the heating time was demonstrated before by Ju and Kilara (1998). As they applied the same time-temperature combination for all protein concentrations at heating, differences in aggregate content/degree of aggregation may be expected. In contrast to Ju and Kilara, in this study and in the study of Le Bon (2001) heating conditions were applied resulting in aggregates with in excess of most of the native proteins participating in aggregate formation.

3.2.3. Electrophoretic mobility

Continuous agarose gel electrophoresis, in the presence of SDS, was used to demonstrate differences in electrophoretic mobility of the WPI aggregates (Fig. 3A). This technique was shown to be very suitable to separate protein (aggregates) with a diameter ranging from 3 (monomeric protein) to approximately 250 nm (Alting et al., 2000, 2002; Vasbinder, Alting, Visschers, & De Kruif, 2002). Since no stacking gel is used, bands are rather diffuse. In addition, the band caused by the aggregates will also be broadened because of polydispersity. In general two bands were observed after electrophoresis of the dispersion of aggregates. One band with the highest electrophoretic mobility corresponding to an unheated solution of WPI (native protein) and another one, with a lower electrophoretic mobility corresponding to the WPI-aggregates. The electrophoretic mobility of the latter clearly decreases with increasing protein concentration at heating, reflecting an increase in aggregate size. As reported before the treatment with NEM had no effect on the electrophoretic mobility (Alting et al., 2000).

3.2.4. Voluminosity and intrinsic viscosity

The dependence of the size of the aggregates on the initial protein concentration was also reflected in

Table 2
The effect of the protein concentration during heating, the cold gelation process and the blocking of thiol groups on the hydrodynamic diameter of WPI aggregates. The aggregates and gels were dissolved and characterized in the SDS-buffer system (final protein concentration 0.5%)

Concentration protein (%)	Before gelation		After gelation	
	Unblocked aggegrates hydrodynamic diameter (nm)	Blocked aggregates hydrodynamic diameter (nm)	Unblocked aggregates hydrodynamic diameter (nm)	Blocked aggregates hydrodynamic diameter (nm)
3	51	51	92	52
4.5	49	50	122	51
6	55	55	301	54
7.5	60	60	305	57
9	67	67	322	63

the voluminosity and intrinsic viscosity of the WPI-aggregates (Table 3). In agreement with the size of the aggregates, both the voluminosity and intrinsic viscosity strongly depended on the initial protein concentration. Vardhanabhuti and Foegeding (1999) also showed recently, that the initial protein concentration was a significant factor in determining the size and/or shape of the aggregates and therefore the intrinsic viscosity of a solution of WPI-aggregates. Britten and Giroux (2001) demonstrated that the intrinsic viscosity in addition to the protein concentration depended on other conditions at heating, namely the calcium concentration and pH. As a general trend it was observed (Britten & Giroux, 2001) that aggregates with a high intrinsic viscosity produced stronger acid-induced cold-set gels. A relationship between the shape of the aggregates and gel strength was suggested.

3.2.5. Cryo transmission electron microscopy

Dispersions of soluble primary aggregates were observed in the cryo-TEM to allow characterization of their 3D morphology in the hydrated (unaltered) native state. This technical approach is based on the vitrification of water by rapid freezing and thereby completely prevent ice crystal formation (Dubochet & McDowall, 1981; Heertje & Paques, 1995). Dispersions of soluble aggregates made of different initial protein concentrations (3–9%) were examined on the morphology of individual aggregates and possible super-aggregate arrangements. A trend of increasing aggregate size from point shaped to linear (coarse irregular helical with uniform strand thickness) shaped of the 3–9% samples was found (Fig. 4, Table 3). The films made of 3% samples showed a small number of aggregates. Both point shaped particles (approx. 7 nm in diameter) and short linear shaped particles (up to approx. 60 nm in length) were observed. Films of the 4.5% samples showed more aggregates, mostly linear (up to approx. 70 nm). The 6% sample showed a number of aggregates comparable to the number found in the 4.5% sample, but only linear aggregates were present (up to approx. 100 nm). The films of the samples of 7.5 and 9% contained only linear aggregates showing a more pronounced curved (flexible)

strand like morphology. The maximum contour lengths found are approx. 130 nm (7.5% protein) and 140 nm (9% protein). The fact that the particle sizes as observed by cryo-TEM appear significantly larger than those observed with DLS can probably be explained by the elongated shape of the aggregates, as also indicated by the intrinsic viscosity data (Section 3.2.4), that is not taken into account in the standard analysis of the light scattering data and by the different sample preparation methods applied.

In addition both samples show super-aggregate arrangements in clusters (Fig. 4C). These clustered arrangements either may have arisen from linear aggregates that become entangled after heating or from branched aggregates formed during the heating step. However, we could not discriminate
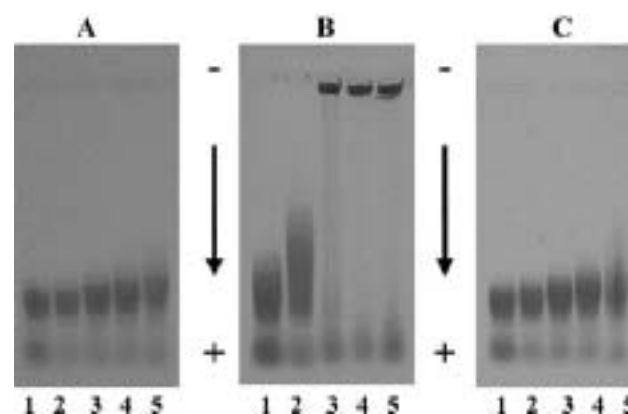

Fig. 3. SDS–agarose gel electrophoresis of WPI-aggregates: the effect of protein concentration (w/w) at heating and blocking of the thiol groups on the molecular weight of the aggregates before and after gelation and resolubilization. Proteins were stained according to the standard Coomassie Brilliant Blue staining method. (A) The electrophoretic mobility of dispersions of aggregates prepared by heating at initial protein concentrations of respectively 3 (lane 1), 4.5 (lane 2), 6 (lane 3), 7.5 (lane 4) and 9% (lane 5) (before gelation). (B) The electrophoretic mobility of unblocked aggregates after gelation and resolubilization. Lanes 1–5 correspond with the initial protein concentrations 3–9% (w/w). (C) The electrophoretic mobility of blocked aggregates after gelation and resolubilization. Lanes 1–5 correspond with the initial protein concentrations 3–9% (w/w).

Table 3
Structural characteristics of WPI-aggregates prepared by heating at different protein concentrations

Protein concentration at heating (%)	Hydrodynamic diameter of WPI-aggregates[a] (nm)	Maximum contour length determined by cryo-TEM (μm)	Intrinsic viscosity of WPI-aggregates (ml/g)	Voluminosity of WPI-aggregates (ml/g)
3	29	60[b]	20	7.3
4.5	29	70[c]	22.8	8.1
6	33	100[d]	28.4	11.0
7.5	44	130[e]	40.7	14.9
9	69	140[f]	47.9	19.0

[a] 0.2% protein aggregates in bidest.
[b] Point shaped (7 nm) and short linear shaped particles.
[c] Mostly linear shaped aggregates.
[d] Only linear shaped aggregates.
[e] Clustering/branching of linear shaped aggregates.
[f] Clustering/branching of linear shaped aggregates.

between these mechanisms on the basis of the electronmicrographs.

3.3. Modification of protein aggregates

In addition to the variation in structural properties, a second variation in type of aggregates was obtained by blocking the free thiol groups present on the reactive WPI aggregates with the sulphydryl reagent NEM. As reported previously (Alting et al., 2000), this modification of reactive thiol groups on the surface of the aggregates was carried out after the heat-treatment step (first step of the process of cold gelation) and did not affect the size of the WPI-aggregates as observed with DLS (Table 2). Treatment with the thiol-blocking agent, NEM, also had no effect on the electrophoretic mobility of the WPI aggregates. In this way WPI-aggregates were prepared which differ in size and in the amount of reactive thiol groups. Blocking of the thiol groups on the WPI-aggregates will prevent chemical cross-linking reactions between aggregates during acid-induced gelation (Alting et al., 2000).

3.4. Characterization of acid-induced cold-set WPI-gels

Cold-set gels were prepared by lowering the pH of the dispersions of either thiol-modified (blocked) or reactive (unblocked) WPI aggregates at ambient temperature to a final pH of approximately 5. As an acidifier, GDL was added. In water, this ester slowly hydrolyses to gluconic acid, causing a gradual reduction in pH and in all cases a regular turbid gel is formed. At a pH near the iso-electric point of the proteins the electrostatic repulsion between the aggregates is strongly reduced and therefore aggregation through non-covalent chemical interactions is promoted (Alting et al., 2002). The final gels were characterized by structural, mechanical and biochemical methods.

3.4.1. Microstructure

The microstructures of the cold-set gels prepared from WPI-solutions heated at different protein concentration (3–9%), but gelled at the same final protein concentration (2%) were visualized by CSLM (results not shown). To the eye, the overall structure was not changed by varying the protein concentration at heating. Quantitative analysis of CSLM images revealed some small variation in the protein filling of the matrix, which could be explained by a small variation in the degree of aggregation, so with the content of protein participating in building the protein network. As reported earlier, the modification of the aggregates with the thiol-blocking agent NEM had no effect on the formation of the microstructure at a μm-length scale (Alting et al., 2000).

3.4.2. Large scale deformation

Although the microstructure of gels made from whey protein heated at different protein concentrations was not significantly different, the mechanical properties clearly were. Fig. 5A shows the force needed to penetrate the different cold-set whey protein gels (final protein concentration 2% protein). The higher the protein concentration at heating, the higher the force needed to penetrate the gel and to break the gel (gel hardness, Bourne, 1978). As observed for different characteristics of the WPI-aggregates, there is a critical protein concentration (4.5%) after which the gel-hardness increases more strongly with the protein concentration at heating. Although the dependence of the gel hardness on the initial protein concentration was in agreement with the observation of Ju and Kilara (1998), they did not observe a critical concentration. We expect that this is caused by differences in the degree of protein aggregation. Based on their results Ju and Kilara concluded that the hardness of cold-set gels of whey proteins is determined by the size and the degree of aggregation. Their conclusion was supported by Mleko (1999), who studied a two-stage heating process.
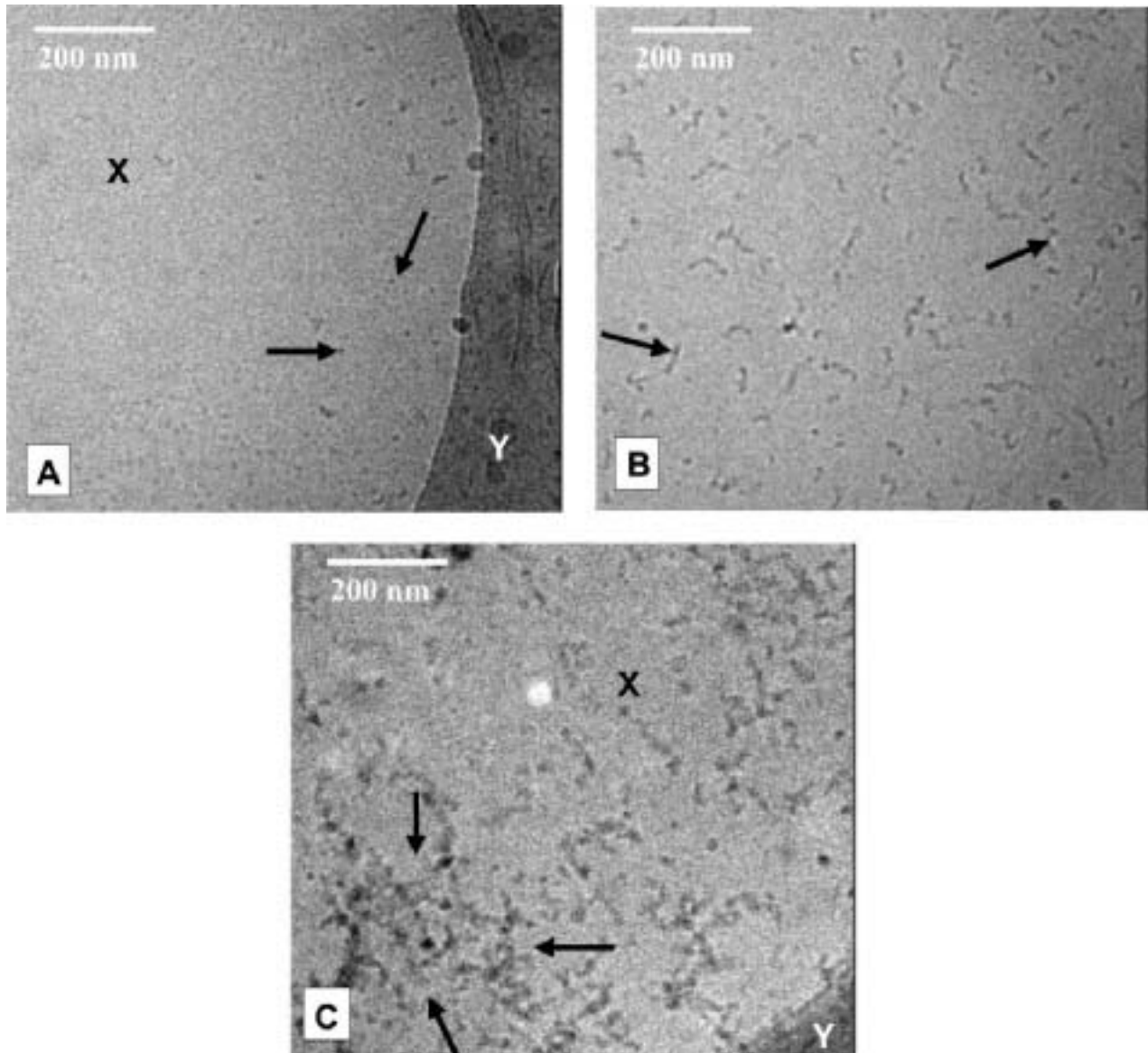
Fig. 4. Cryo-TEM observation of primary aggregates prepared by the thin film technique that allows characterization of the 3D morphology in the hydrated (unaltered) native state. (A) The image shows the 'whole area' (X) and the 'carbon film area' (Y) of the whole carbon film part of the specimen. The whole area contains the thin film of the sample and is the site of TEM observation. The arrows indicate the 'point shaped particles'. Image taken from sample of 3% initial protein concentration. (B) The image shows the whole area of the specimen and arrows indicate two of the many linear protein aggregates present. Image taken from sample of 6% initial protein concentration. (C) The image shows the whole area (X) and the arrows enclose a clustered arrangement of linear aggregates.

From previous work the importance of disulfide bonds formed in the second step of the process of cold gelation was recognized. Therefore, to solely study the effect of structural properties, as the aggregate size, on gel hardness, thiol-blocked aggregates were applied. The gelation kinetics of thiol-blocked WPI-aggregates and the microstructure formed after addition of GDL did not differ (Alting et al., 2000, this study). Fig. 5B shows the force needed to penetrate gels made from thiol blocked aggregate solutions differing in size. It is clearly seen that these gels are much weaker than gels made from unblocked aggregates. The effect of the size of the aggregates on the gel hardness is only a minor effect compared to the effect of thiol groups on the mechanical properties of cold-set gels.

3.4.3. Determination of disulphide bonds

After gelation, gels made from blocked and unblocked aggregates were dissolved in a buffer containing SDS, which brakes up physical bonds, but leaves covalent bonds intact. Comparison of Fig. 3A and B, clearly shows that the electrophoretic mobility of resolubilized aggregates decreases (aggregate size increases) with increasing protein concentration at heating. This clearly demonstrates the formation of additional inter-particle disulfide bonds
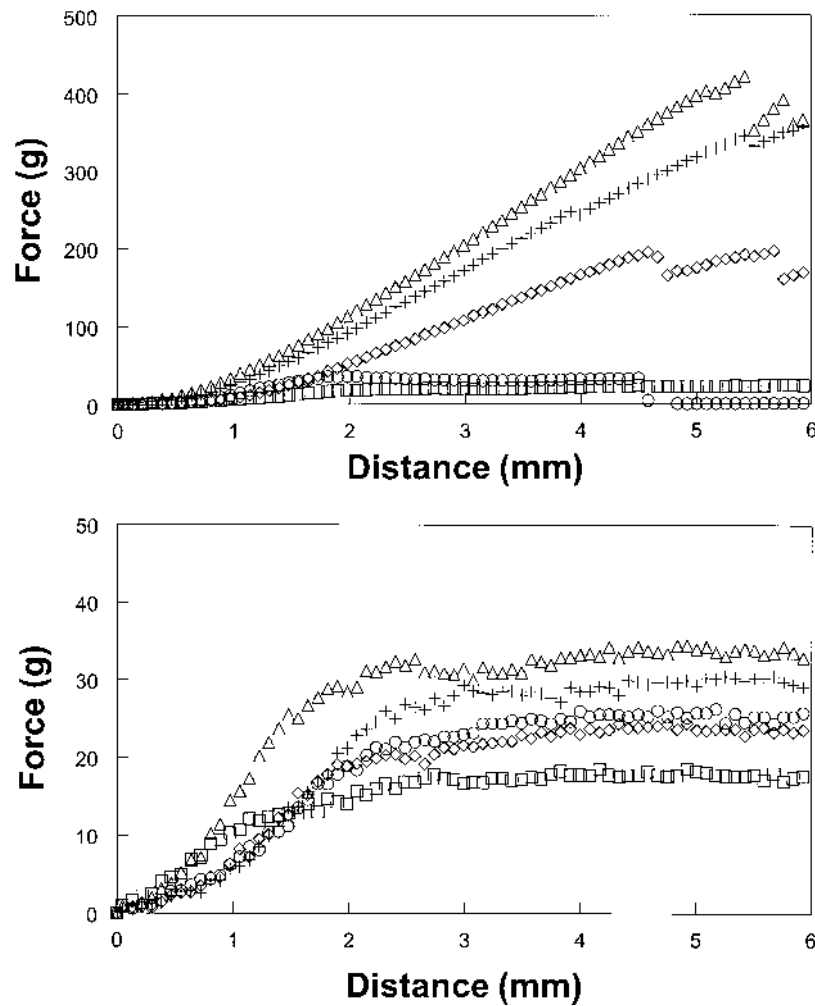
Fig. 5. Force-distance curves showing the effect of protein concentration (w/w) at heating (A) and the additional thiol blocking (B) on the hardness of acid-induced gels (squares, 3%; circles, 4.5%; diamonds, 6%; crosses, 7.5% and triangles, 9%).

during cold gelation. Also this figure demonstrates that above an initial protein concentration at heating of 4.5% much more disulfide cross-links were formed, resulting in large disulfide cross-linked protein structures which could not enter the agarose–matrix anymore. As expected, no changes in electrophoretic mobility were observed for the resolubilized gels prepared from blocked WPI-aggregates (Fig. 3C). In addition, differences in aggregate size, induced during gelation, were characterized by DLS (Table 2). Note that before gelation, the aggregates solubilized in SDS-buffer seem larger at the lower initial protein concentration than solubilized in water (Table 3). This is probably caused by swelling of the aggregates after binding negatively charged SDS-molecules (repulsion). After gelation and resolubilization of the gels comparable results were found to that of the SDS–agarose electrophoresis. No significant changes in the hydrodynamic diameter of the blocked aggregates after gelation was shown, in contrast to a large effect on the hydrodynamic diameter of the unblocked aggregates. Again a larger increase in size is observed at initial protein concentrations higher than 4.5%.

3.4.4. Importance of thiol groups

Recently, we demonstrated that covalent interactions (disulfide bonds) do not significantly contribute to the initial acid-induced aggregation and network formation process of WPI-aggregates. Surprisingly, formation of covalent disulfide bonds was observed under acidified conditions once a protein network was formed. This delayed formation of disulfide bonds with time was also observed for high-pressure induced gels of β-lactogobulin (Dumay, Kalichevsky, & Cheftel, 1998) and acid milk gels (Vasbinder et al., 2002). The formation of disulphide bonds at low pH was contributed to a large increase of the effective protein concentration in the protein network and had a large effect on the mechanical properties of acid-induced gels (Alting et al., 2000). As reported earlier for heat-induced gels of whey proteins (Monahan, German, & Kinsella, 1995; Shimada & Cheftel, 1989), the disulfide cross-links were primarily of the SH/S–S-interchange type as also in our system the amount of thiol groups before and after gelation (and resolubilization) did not significantly differ (results not shown).

It is expected that all aggregates will have potential cross-linking possibilities, since significant amounts of thiol groups were present in all cases. Typically individual aggregates will expose 10–1000 thiol groups. The reactivity of the thiol group, which is strongly pH-dependent, probably determines the amount of inter-particle cross-links formed. Alting et al. (2002) demonstrated by the use of succinylated protein aggregates (lowered iso-electric point) that at pH values below 3.5 no disulfide cross-links are formed during the gel state. Assuming a pK of 8.3 for thiol deprotonation, it follows that at pH 5 only 1:3160 sulphur-groups is de-protonated and therefore able to initiate thiol/disulfide exchange reactions. Below an initial protein concentration of 4.5%, the number of thiol groups may fall below a critical value at which the disulfide cross-linking cannot result in a space-filling network of additional covalent bonds between the aggregates.

Previously we determined the dependence of the gel hardness on the relative number of thiol groups. In that study, the number of thiol groups was varied by chemically blocking of the thiol groups. Here, the number of thiol groups was varied by differences in the heating conditions. In Fig. 6 the hardness of gels prepared from dispersions of aggregates differing in structural properties, but more importantly in the number of thiol groups are compared with the previous obtained results. As in the previous study, aggregates prepared by heating at an initial protein concentration of 9% were regarded as 100% unblocked aggregates (see also Fig. 2). The concentration of thiol groups determined in dispersions of aggregates prepared by heating at lower protein concentrations are expressed as a percentage of that (see also Table 2). By varying the number of thiol groups either by chemical treatment or by changing the heating conditions we demonstrated that there is a strong and identical dependence between the final hardness of cold-set gels and the number of thiol groups present after the first step of the cold gelation process (Fig. 6).
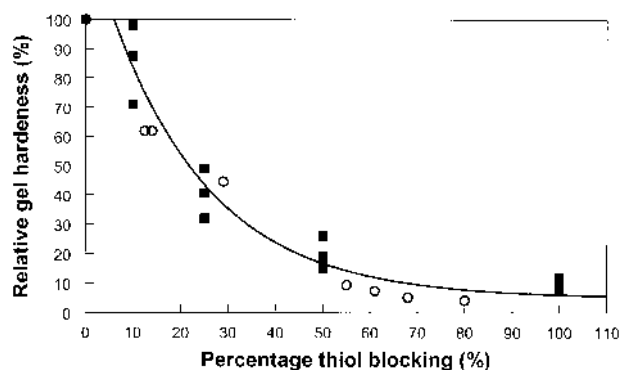

Fig. 6. Dependence of the relative gel hardness on the relative number of thiol groups present on the soluble aggregates. The closed squares represent the hardness of gels prepared from chemically thiol-blocked WPI-aggregates. The open circles represent the variation in the number of thiol groups caused by differences in the heat treatment. The line drawn is to guide the eye.

4. Conclusions

Variation of the protein concentration at heating resulted in formation of protein aggregates with clearly different structural and chemical characteristics. Under the chosen heating conditions differences in the degree of protein denaturation were excluded. In addition to differences in hydrodynamic diameter size, electrophoretic mobility, geometric characteristics, voluminosity and intrinsic viscosity, the number of accessible thiol groups clearly differed. Also the mechanical properties of cold-set gels depended strongly on the protein concentration at heating. Although gel hardness seemed to correlate with the different structural aggregate features as reported before in literature (Britten & Giroux, 2001; Ju & Kilara, 1998), it could for the most part be cancelled by blocking of the thiol groups. Application of thiol-blocked protein aggregates enabled us to make a distinction between the effect of structural- and chemical-properties of the aggregates. Formation of larger disulfide cross-linked protein structures paralleled with the increase in gel hardness and dominated over the effect of structural characteristics on mechanical properties of cold-set gels. Although, in the absence of reactive thiol groups only a minor effect of structural properties on gel hardness was found, they seemed to be correlated. This correlation will be subject of further research. In addition, the effect of the presence of native non-aggregated protein on the final gel properties can be excluded, since in our gel-experiments most protein ($>95\%$) participated in the formation of a protein network. Therefore, we can conclude that the hardness of cold-set whey protein gels is determined by the number of thiol groups rather than by the size of the aggregates or other structural features.

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Chapter 5

Cold-set globular protein gels; interactions, structure and rheology as a function of protein concentration

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Cold-Set Globular Protein Gels: Interactions, Structure and Rheology as a Function of Protein Concentration

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We identified the contribution of covalent and noncovalent interactions to the scaling behavior of the structural and rheological properties in a cold gelling protein system. The system we studied consisted of two types of whey protein aggregates, equal in size but different in the amount of accessible thiol groups at the surface of the aggregates. Analysis of the structural characteristics of acid-induced gels of both thiol-blocked and unmodified whey protein aggregates yielded a fractal dimension (2.3 ± 0.1), which is in line with other comparable protein networks. However, application of known fractal scaling equations to our rheological data yielded ambiguous results. It is suggested that acid-induced cold-gelation probably starts off as a fractal process, but is rapidly taken over by another mechanism at larger length scales (>100 nm). In addition, indications were found for disulfide cross-link-dependent structural rearrangements at smaller length scales (<100 nm).

INTRODUCTION

Preparation of globular protein gels can be viewed as consisting of three distinct steps: denaturation, aggregation, and gelation. In a typical heat-set or thermotropic gel, these processes are intertwined and occur simultaneously during a one step heating process. In cold-set gels, denaturation and aggregation are separated from the gelation step (*1*). In this procedure, protein aggregates are first prepared by heating a solution of native proteins at a pH well above the isoelectric point of the protein and in the absence of salt. This results in the formation of repulsive aggregates. Second (after cooling), an acid-induced cold-set gel is formed by gradually lowering the pH to the isoelectric point of the protein aggregates (*2*). In this way, aggregation and gelation take place sequentially, which makes it possible to study the direct relationship between aggregate and final gel properties. Also, complications that arise from the use of protein mixtures are less troublesome, because these mainly influence the primary denaturation and aggregation kinetics. Both heat- and cold-set gels of globular proteins are used to improve the texture of dairy products, fresh meat, fresh fish, and surimi. To improve the application of these ingredients, it is important to establish relationships between consumer perception, texture, and mouthfeel on one hand and rheological properties of the protein gels on the other hand. The rheological properties of gels are a complex function of the ingredient composition, microscopic properties, and mesoscopic structures of gel matrixes. Although a direct relationship between rheological parameters and consumer perception seldom exists, it may be feasible to predict how practically relevant food properties scale with the amount of protein.

In the literature, different models exist that describe the scaling behavior of gel properties with the protein concentration. In the so-called cascade model, rheological scaling behavior is related to polymer−polymer interaction and the formation of junction zones (*3*). The concept of fractals (*4*) provides an alternative approach to explaining the scaling behavior of the elastic properties of the aggregate network. In this model, the geometric structure is scale-invariant. A single parameter D, called fractal dimensionality, describes the geometric properties of the network for the typical length scales at which the random aggregation mechanism occurs. Several theories have been developed to predict the scaling behavior of micro-structural properties and rheological properties with the protein concentration on the basis of the fractal dimension, in both heat- and cold-set gels (*5*, *6*, *7*, *8*). However, these theories seem not to be applicable in all cases (*9*, *10*). Verheul and Roefs (*11*) clearly demonstrated for heat-set gels of whey protein isolate (WPI) that only a fraction of the protein is aggregated and available for the building of a protein network at the point when the microstructure is formed. In contrast to heat-induced gelation,

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with acid-induced cold gelation all the protein is in its aggregated form and available to participate in the protein network from the start of the gelation. Therefore, it may be expected that cold-set gels will better satisfy the conditions for fractal aggregation than heat-set gels do.

The fractal dimension of a particle gel can be derived from different gel properties: the wavelength dependence of the turbidity (6, 12), correlation analysis of confocal scanning laser microscope (CSLM) images (6, 13), and also from the concentration dependence of the permeability coefficient (6, 13, 14) or the storage modulus (6, 13, 15). Turbidity, microstructural parameters, and permeability are, in general, not influenced by the interactions between the particles. Rheological properties (storage modulus) on the other hand depend on both the geometry and the connectivity of the protein network.

Here, we aim to identify the contribution of structural and interaction parameters to the rheological properties of cold-set gels of WPI. Previously, we have developed a cold-gelation system, consisting of two types of WPI aggregates, equal in size but different in the amount of accessible thiol groups at the surface of the aggregates (16). We demonstrated that the initial microstructure of the gel is mainly determined by noncovalent interactions and is not affected by modifying the amount of disulfide bonds. This is in contrast with the mechanical properties, which can be tuned through changing the amount of reactive thiols present during gelation. Because it is possible with this model system to precisely determine and modify the properties of the WPI aggregates, it should be feasible to identify the individual contributions of covalent and noncovalent interactions to the rheological behavior of protein gels and their scaling with the protein concentration.

In this study, we investigated the scaling behavior of geometrical and rheological properties of acid-induced cold-set WPI-gels. Two different gel systems were characterized: gels made from WPI aggregates with and without thiol-blocking treatment. The results are discussed on the basis of both the gel microstructure and the rheological properties of the gels and are linked to the mechanism of aggregation.

MATERIAL AND METHODS

Reagents and Chemicals. D-gluconic acid lactone (GDL), 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), sodium dodecyl sulfate (SDS), and N-ethylmaleimide (NEM) were obtained from Sigma Chemicals (St. Louis, Mo. USA). The whey protein isolate (WPI) Bipro was obtained from Davisco Foods 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 is 97.5%, and it further contains lactose (0.5%) and ash (2%) (17).

Preparation of Reactive WPI Aggregates. A 9% solution of WPI aggregates was prepared by heating at 68.5 °C (16). The pH after solubilization and heating was 7.2. The solution of WPI aggregates was diluted with filtered (0.22 μm; Millex-GV, Millipore Corporation, Bedford, MA) double-distilled water to different concentrations (0.5−9%) (w/w) and stored at 4 °C. Before storage, the headspace was filled with nitrogen to prevent oxidation of the thiol groups. Sodium azide (0.02% final concentration) was used as a preservative. This heat treatment of a 9% (w/w) WPI solution resulted in a "stable" dispersion of reactive WPI aggregates with a hydrodynamic diameter of 70−80 nm (in a buffer containing SDS), with in excess of 95% of the native proteins participating in aggregate formation (16). Under the same conditions, a pure β-lactoglobulin preparation produces aggregates with both comparable size and amount of thiol-exposure (2).

Blocking of Free Thiol Groups. Reactive aggregates were treated with NEM at various concentrations (0−5 mM), depending on the protein concentration. The efficiency of the blocking reaction was determined by the use of the Ellman's reagent as previously described (16). After 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. As reported before (2, 16), this modification did not change the size of the WPI aggregates.

Preparation of Acid-Induced Gels. GDL was added as a powder to the WPI solutions to induce cold gelation at ambient temperature. In water, GDL slowly hydrolyses to gluconic acid, causing a gradual reduction in pH toward the isoelectric pH of the protein aggregates. The total amount of GDL required depends on the protein concentration (18). Different amounts had to be added to reach a pH of around 5 after approximately 24 h of incubation at ambient temperature. Amounts of 0.06%, 0.10%, 0.12%, 0.14%, 0.23%, 0.42%, and 0.63% (w/w) were added to 0.5, 1, 1.5, 2, 3, 6, and 9% protein-solutions, respectively, to reach a pH value of around 5. The acidification induced gelation of the dispersion of WPI aggregates.

Solubilization of Gels. To determine the size of the aggregates after gelation, gels were solubilized with 3 parts of 20 mM Bis-Tris buffer (pH 7.0) 5% SDS and were held at ambient temperature while constantly stirred. After overnight incubation, no gel particles could be observed with a standard microscope (at 400 × magnification).

Dynamic Light-Scattering Experiments. Dynamic light-scattering (DLS) experiments were performed as outlined by Verheul et al. (19). Before analysis, samples were filtered through a low-protein-binding membrane (5 μm; Millex-SV, Millipore Corporation).

Turbidity Measurements. Turbidity measurements were performed at 25 °C on a Cary 1E UV−Vis spectrophotometer (Varian Nederland BV, The Netherlands) equipped with a temperature controller. The turbidity was measured with 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 (11) and based on a method of Van Dijk and Walstra (20). Briefly, a 9% (w/w) solution of WPI aggregates was diluted to different concentrations (0.5−9%) with double-distilled water. Gels were prepared at ambient temperature in open glass tubes (25 cm × 3.7 mm). Approximately 24 h after the addition of GDL, they were placed in a measuring device to monitor the flow of solvent (double-distilled water, pH 5) under hydrostatic pressure. Mean permeability coefficients were determined by measuring the flux of solvent with time of 12 different tubes, using Darcy's law. Within experimental accuracy, the permeability coefficient of WPI gels did not change with time during the experiment.

Assuming that the structure of the network of the cold-set WPI gels can be considered as fractal, the permeability (B) and the volume fraction (φ) or the concentration of the WPI aggregates are related through the following equation derived by Bremer (6):

$$B = (a^2/K)\varphi^{2/(D_f - 3)} \tag{1}$$

where K is a constant, D_f is the fractal dimension, and a is the radius of the primary particles.

CSLM. 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% (w/v) Rhodamine B. The 568 nm laser line was used for excitation, inducing a fluorescent emission of Rhodamine B, detected between 600 and 700 nm.

The fractal dimension of the CSLM images was obtained using a box-counting algorithm, which has the advantage that it does not assume a linear relation between the intensity of the CSLM micrographs and the local protein concentrations. Digitized CSLM images were first thresholded at the mean intensity and 20% above and below this value to determine the boundary between protein and solvent. Then, a closing operation (dilution followed by erosion) was performed to fill the gaps between closely spaced pixels in the image. This operation was followed by an opening operation (erosion followed by dilation) to remove small dark regions in the background of the images (21). Subsequently, a square mesh of a certain size L was laid over the outline of the objects on the digitized images. The number of mesh boxes, $N(L)$, that contain

Table 1: Exponent A and B in the Equations $G' \propto \varphi^A$ and $\gamma_0 \propto \varphi^B$, According to Bremer, 1992 and Shih et al. (1990)[a]

A	B	reference
$2/(3-D_f)$	0	(straight strands) Bremer, 1992
$3/(3-D_f)$	$1/(D_f-3)$	(curved strands) Bremer, 1992
$(3+x)/3-D_f$	$-(1+x)/(3-D_f)$	(strong-link regime) Shih et al., 1990
$1/(3-D_f)$	$1/(D_f-3)$	(weak-link regime) Shih et al., 1990

[a] The relationships between G', γ_0, and φ given by Mellema (2000) and Wu and Morbidelli, (2001) are extensions of the models given.

part of the outline of the objects was counted. The fractal dimension, D, of the protein aggregates in a two-dimensional projection was calculated from the slope of the double logarithmic plot for $N(L)$ against L, considering the relationship between the parameters

$$N(L) \sim L^{-D} \qquad (2)$$

Determination of D on the basis of the contour lines of the geometric structures circumvents the problem that the relation between observed fluorescence intensity and actual protein concentration is probably not linear. All operations were performed with the public domain program ImageJ (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/ij/). The fractal dimension of protein aggregates in three dimensions D_f, can be calculated from the following equation (6):

$$D_f = D + 1 \qquad (3)$$

Rheological Measurements. Small-amplitude oscillatory measurements were made with a Carri-Med CLS[2] 500 rheometer (TA Instruments, Leatherhead, UK) using a conical concentric cylinder measuring unit (inner radius 8.60 mm, outer radius 9.33 mm). Immediately after the addition of GDL, samples were brought into the measuring unit and covered with a thin layer of paraffin oil to prevent evaporation. All experiments were conducted in oscillation at a frequency of 1 rad/s (0.159 Hz). First a strain sweep, $G(\gamma)$, was recorded, and the part of the strain sweep where the storage modulus was independent of strain was taken as the linear region. The formation of a gel network for both the blocked and unmodified aggregates at different protein concentrations was followed by the development of G' and G'' with time. Gelation and aging of the gels were monitored at 25 °C. Because the network formation during gelation will probably be more sensitive to deformation, a strain at the beginning of the linear region was chosen (1%). The frequency sweeps were performed within the linear region. In these experiments, the frequency was varied from 0.01 to 20 Hz at a temperature of 25 °C.

In an ideal network model, all particles are arranged in a statistical network of chains that all contribute to the same extent to the rigidity of the network. Because it is not generally found that the rigidity of a gel is proportional to the particle concentration, aggregate network models have been developed, and the concept of fractal geometry was introduced. In these models, a particle gel is made up of fractal flocs. The flocs themselves are made up of strands of primary particles (6). Different aggregate network models are proposed in the literature to translate rheological data into information about the interactions between aggregating particles, the fractal dimension, and the aggregation mechanism (5, 6, 7, 8). In general

$$G' \propto \varphi^A \qquad (4)$$

$$\gamma_0 \propto \varphi^B \qquad (5)$$

The storage modulus (G') and the limit of linearity (γ_0) will strongly depend on the way the clusters are linked to each other. The exponent A in equation 4 and B in equation 5 will therefore vary with the particle gel system studied (**Table 1**).

RESULTS AND DISCUSSION

**Microstructural Measurements.** CSLM and permeability measurements were made at protein concentrations (WPI
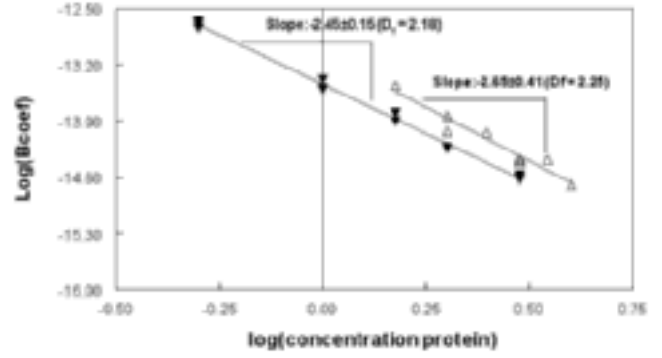
Figure 1. Double logarithmic plot of the protein concentration dependence on the permeability coefficient (m^2) of acid induced gels of blocked (open symbols) and unmodified WPI aggregates (concentration in percent weight/weight) (closed symbols).

aggregates) ranging from 0.5 to 9%. Gelation, under the conditions applied, occurred at a minimal protein concentration of 0.5% (9% diluted to 0.5%) for unmodified aggregates. Gels made from thiol-blocked aggregates were much weaker. A protein concentration of 1.5−2% was needed to form a gel that could withstand gravity and handling. In all cases, turbid gels were formed. Gels made from blocked aggregates showed some syneresis and some spontaneous gel fracture after 16 h that increased with time.

Figure 1 shows that the permeability of the gels (B_{gel}) decreased with increasing concentration of WPI aggregates. This indicates that the pores in the gel are smaller when more protein is present. The permeability of the different acid-induced cold-set WPI-gels (B_{gel}) did not change between 24 and 72 h after addition of GDL. At protein concentrations higher than 4%, the flow of liquid through the gel matrix was too low to perform reliable permeability measurements. A linear relationship was observed between B_{gel} and the WPI concentration in a double logarithmic plot (**Figure 1**). B_{gel} decreases by almost 2 orders of magnitude between a protein concentration of 0.5 and 3%. The lines drawn are fitted lines, the slope of which corresponds to a power law with an exponent of around −2.5 and is not significantly different for gels of both the unmodified and blocked aggregates (95% confidence limit).

The results indicate that, within the error of this experiment, the scaling behavior is the same for both types of gels. It must be noted that measurements at other protein concentrations revealed a small difference in permeability between gels made from solutions of blocked aggregates and those made from unmodified aggregates. This is in contrast to an earlier observation (16), where the permeability was determined at a single protein concentration of 2% and was not significantly different. The difference in pore size can probably be explained by a difference in the rate of structural rearrangements during aggregation and gelation. To further investigate this, acid-induced gelation was followed by measurement of the turbidity with time (**Figure 2**). The initial rapid onset of the turbidity took place at the same pH (16) and reflected the formation of a protein network. Once the network was formed, differences were observed for the two types of aggregates in the steady increase of the turbidity with time, suggesting different kinetics of structural rearrangements.

As a consequence of the acidification, electrostatic repulsion decreases and tenuous clusters of aggregates are formed by physical interactions (2). Such open clusters of aggregates are thermodynamically unstable in comparison with a denser cluster, in which more physical bonds can be formed (6). In the case of
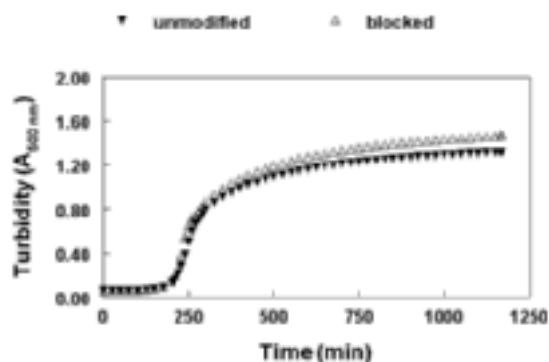
Figure 2. Development of the turbidity of solutions of blocked (open symbols) and unmodified aggregates (closed symbols) (protein concentration 2%) during acid-induced gelation.
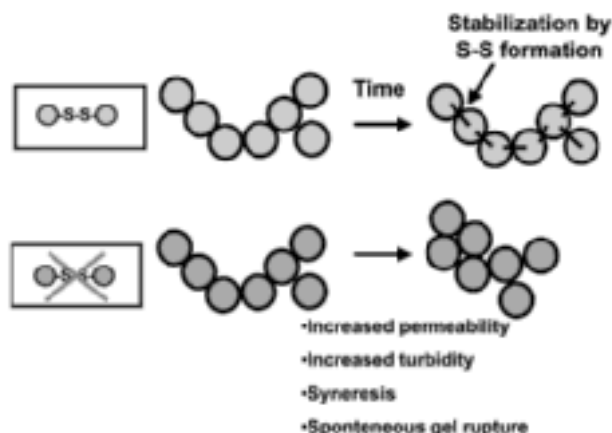

Figure 3. Schematic representation of the occurrence of structural rearrangements.

unmodified aggregates, the initial tenuous structure of these clusters can be (partly) stabilized by the formation of additional covalent disulfide bonds. Blocked aggregates are not able to form these additional stabilizing bonds. As a result of the intrinsic instability, rearrangements can occur much faster, which leads to locally denser clusters and larger pores. This will result in a more turbid gel and a more permeable microstructure (**Figure 3**), which is in line with our observations. As far as we know, no other observations have been reported of a direct relationship between disulfide bond formation and structural rearrangements. As we observed, the rearrangements also cause more syneresis.

The permeability results were also compared with the work of Verheul and Roefs (*11*, *14*). In their studies, heat-induced gels were prepared from WPI at the same heating temperature as applied in our first step of the cold-gelation process, but at other pH values and at higher concentration of salt. By application of the cold-gelation method, gels can be prepared with the same permeability characteristics but at a much lower protein concentration than with heat-induced gelation (0.5 and 3%, respectively).

CSLM images were made from gels prepared with the two types of WPI aggregates for five different protein concentrations (Results not shown). The protein network structure of both types of gels appeared coarser, having larger pores at lower protein concentrations. At a protein concentration of 1%, a thin network of threaded protein material is observed that encloses large holes (3 μm) filled with solvent. Increasing the protein concentration results in smaller, but to the eye similar, structures. At the highest concentrations (>6%) the structures become too small
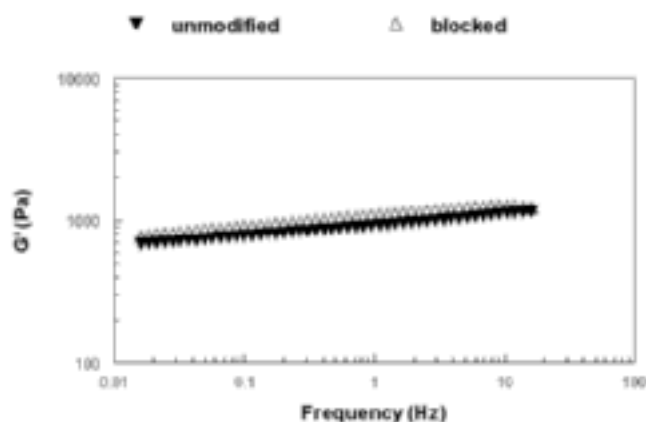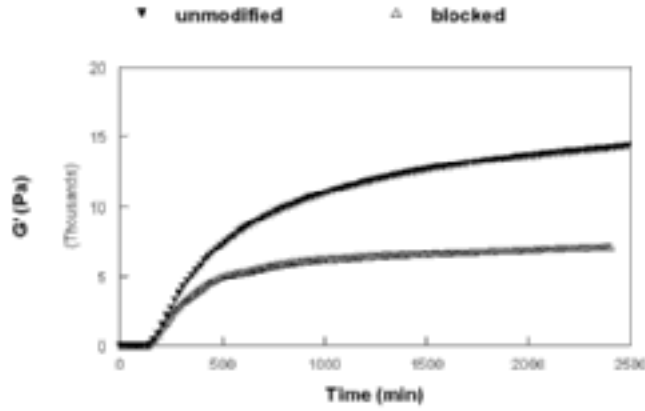

Figure 4. Frequency dependence of the storage modulus (G') of acid-induced WPI gels at a protein concentration of 2% (frequency sweep). Blocked aggregates (open symbols) and unmodified WPI aggregates (closed symbols).

to be fully resolved by the microscope (<0.5 μm). Thus, a more compact network, with smaller pores, was formed when the concentration of protein was increased. There appeared to be no significant differences between the images of the gels prepared from blocked or unmodified aggregates at a protein concentration ranging from 1 to 9%. This is in agreement with our previous observation that blocking the thiol groups on the WPI aggregates did not result in a different microstructure (μm length scale) of the cold-set gel at a protein concentration of 2%, as determined by CSLM (*16*) and small angle light scattering (De Hoog and Alting, unpublished results). Therefore, we conclude that the differences in structural rearrangements occur on a length scale below the resolution of this technique (<200 nm).

Small and Large Deformation Measurements. In contrast to the geometrical gel properties discussed above, larger differences between the two types of aggregates may be expected in rheological measurements, as the rheological properties of the gels studied will depend on both the microstructure of the gel and the interactions between the aggregating particles. The mechanical properties of a gel were monitored during gelation using nondestructive, dynamic measurements. Only the results of G' are presented, because G' and G'' developed simultaneously with time, and the elastic component was more dominant than the viscous component. The elastic behavior of the gels also finds expression in the frequency dependence of the storage modulus (**Figure 4**). Forty hours after the addition of GDL, the frequency dependence of G' was determined for both types of aggregates at a protein concentration of 2%. The dependence of G' on the frequency was the same for both type of aggregates. The storage modulus of purely elastic materials is frequency independent. However, food gels usually have a small contribution of the viscous component, G'', resulting in frequency-dependent G' values reflecting relaxation of these viscous components (*22*). Acid-induced cold-set gels also show this viscoelastic behavior, with G' increasing slightly with increasing frequency.

Figure 5 shows that the initial increase in the storage modulus for gels prepared from unmodified and blocked aggregates did not differ. This is in agreement with our previous observation that the initial kinetics of gelation were not disturbed by blocking the thiol groups (*16*, *23*). Over time, the storage moduli of the gels from the blocked aggregates (only physical interactions) reach a plateau value, whereas for the moduli of gels prepared from the unmodified aggregates, it takes a much longer time

Figure 5. Development of the storage modulus (G') for a 6% (w/w) solution of WPI aggregates (time sweep) (closed symbols unmodified aggregates, open symbols blocked aggregates).

Table 2: Time Dependence of Formation of Disulfide-Linked Aggregates during Acid-Induced Cold Gelation of a 2% (w/w) Solution of WPI Aggregates

|	Gel prepared from unmodified aggregates[a]		Gel prepared from blocked aggregates[a]	
time[b] (h)	diameter (nm)[c]	intensity (cps × 1000)[c]	diameter (nm)[c]	intensity (cps × 1000)[c]
0	83 ± 1	78	82 ± 1	96
16	167 ± 3	193	83 ± 1	96
24	451 ± 6	471	85 ± 1	95
40	465 ± 11	485	86 ± 1	101
48	512 ± 10	530	84 ± 1	97

[a] Gels were dissolved in SDS-buffer system (1:3)(final protein concentration 0.5%). [b] Time after addition of GDL. [c] Determined by DLS (see Material & Methods section). Errors represent the standard error of the cumulant fits within one measurement.

until a plateau value is reached. In this case, as well as physical interactions, formation of additional disulfide bonds is to be expected. **Table 2** clearly shows that with time, disulfide-linked aggregation led to an increase in aggregate size observed after solubilization of the gels (2% (w/w) protein). Pretreatment of the aggregates with a thiol-blocking agent prevented the formation of larger disulfide-linked aggregates. After addition of GDL, no increase in the apparent diameter of thiol-blocked aggregates with time as determined by DLS was observed.

To predict the plateau G'(inf) values at infinite times, we fitted both first-order reaction kinetics and (irreversible) second-order kinetics (24) to the cure curves. We found negligible differences in the obtained G'(inf) between the two types of fits, so we cannot draw conclusions about the actual kinetic mechanism of gelation from these cure curves. However, because both types of fits display significant differences between the unmodified and blocked aggregates, we further analyzed the G'(inf) values as a function of protein concentration for these two systems. From **Figure 6**, it can be seen that the storage modulus (plateau-value) of the gels increased with increasing protein concentration (from 1 to 9% (w/w) protein). The values of (d log G')/(d log C) of gels prepared from blocked aggregates (only physical interactions) or prepared from unmodified aggregates (both physical and covalent interactions) were significantly different irrespective to the type of fit applied. In the absence of thiol groups on the WPI aggregates, this led to a (d log G')/(d log C) of 2.05 ± 0.18. (95% confidence limit). The lines drawn are linear fits to the experimental points, and the line is steeper for
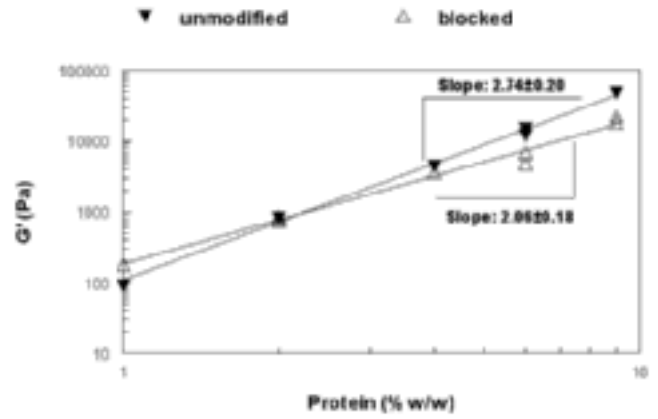
Figure 6. Protein concentration dependence of the storage modulus (G') of acid-induced WPI gels (closed symbols unmodified aggregates, open symbols blocked aggregates).
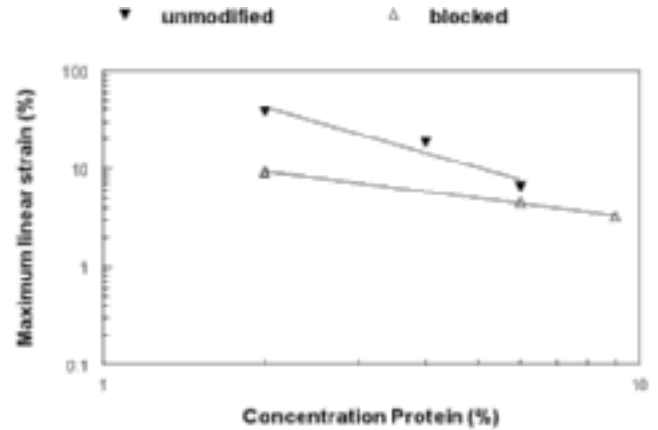
Figure 7. Maximum linear strain of acid-induced WPI gels at different protein concentrations (strain sweep), determined 40 h after the addition of GDL (closed symbols unmodified aggregates, open symbols blocked aggregates).

the unmodified WPI aggregates, with a slope of 2.75 ± 0.20 (95% confidence limit).

Surprisingly, at protein concentrations lower than 2% (w/w) the G' plateau values were higher for cold-set gels prepared from blocked aggregates. As for the microstructural observations, this can be explained by the occurrence of structural rearrangements during the initial stage of the gelation process, resulting in denser packing and more physical interactions and, therefore, higher G' values. At higher protein concentration, this process is frustrated and dominated by the formation of disulfide bonds (**Figure 3**).

Forty hours after the addition of GDL, the maximum linear strain was determined for both types of aggregates (2−9% protein). The maximum linear strain decreased only slightly with increasing protein concentration (**Figure 7**). The maximum linear strain of the unmodified aggregates appears to be more dependent on the protein concentration, and the values of the unmodified aggregates at the different protein concentrations were higher than the values of the blocked aggregates. Strain sweep experiments performed on gels that were cured for 24 h confirmed these trends (data not shown).

The rheological results support the idea that the first stage of the cold-gelation process is driven by physical interactions (2, 16). Gelation of the blocked aggregates can be considered as a one-stage process. Only physical interactions were involved in the gelation process leading to the formation of a space-filling
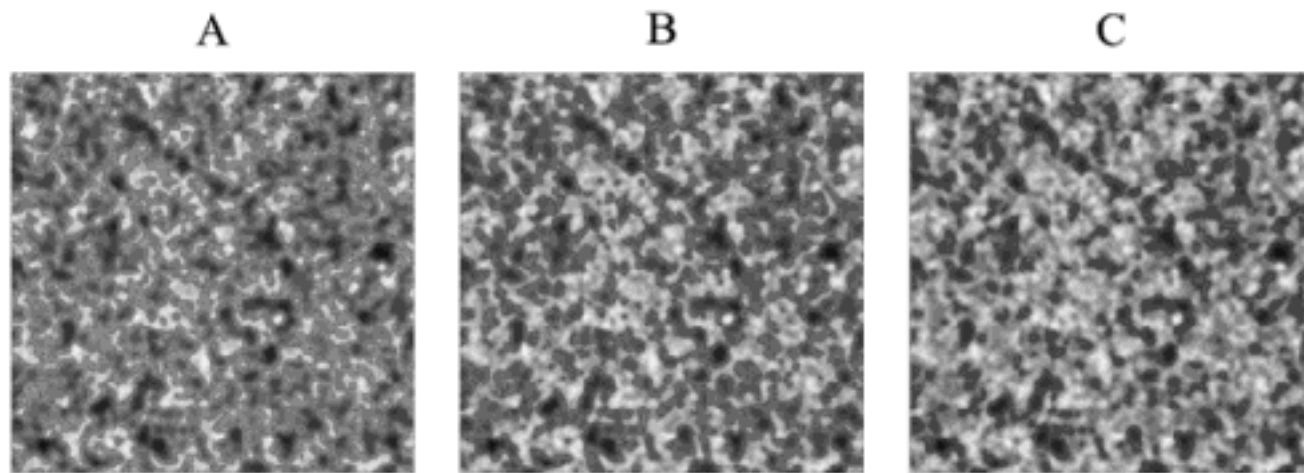
Figure 8. Digitization of a CSLM image of a 2% WPI-gel (unmodified aggregates) at three different threshold values, 30% (A), 50% (B) and 70% (C). The images represent a total surface of $20 \times 20 \ \mu$m.

gel network. No increase in disulfide-linked aggregation with time was observed. In contrast, the gelation process of unmodified aggregates can be considered as a two-stage process. The first stage represents the formation of the gel by physical aggregation, the second stage represents an increase in the gel stiffness and hardness by formation of additional covalent bonds between the structural elements of the gel. This is reflected by a time-dependent increase in G' and hardness and by the formation of larger disulfide-linked aggregates. The difference in the type of gelation process, one- or two-stage, leads to different mechanical properties at the same protein concentration. At lower protein concentrations (<2% (w/w)), the stabilizing role of the disulfide bonds formed is less pronounced, allowing the occurrence of structural rearrangements.

Fractal Nature. If it is assumed that the aggregation process leading to a space-filling network proceeds as a fractal aggregation process, the difference in slope of the double logarithmic plots of both structural and rheological measurements should give insight into the different interactions between the aggregating particles, relating macroscopic to microscopic properties. From the slope of the permeability versus the protein concentration in a double logarithmic plot, a fractal dimension of 2.2 ($\pm$ 0.1) was found for the acid-induced cold-set gels. The fractal dimension calculated suggested a reaction-limited aggregation mechanism (*25*). A value between 1.8 and 2.0 would have suggested a diffusion-limited type of aggregation mechanism. These results were in good agreement with the results from the box-counting algorithm applied to the digitized CSLM-images (contour representation) (**Figure 8**) (fractal dimension of 2.3 $\pm$ 0.1) and with data from other types of cold-set gels reported in the literature (see below). An important observation was that the different imposed thresholds for determining contours did not greatly influence the value of the fractal dimension.

Fractal geometry has been previously used to characterize structures of salt- or acid-induced protein gels (*6, 13, 26*). For GDL-acidified and microbial-acidified milk, a fractal dimension ranging from 2.3 to 2.4 was found by quantitative analysis of CSLM images and by permeability analysis performed at different protein concentrations (*6, 13*). For salt-induced cold-set gels of WPI, a fractal dimension ranging from 2.3 to 2.6 was found depending on the concentration of salt added (*26*).

Thus, scaling behavior of microstructural parameters yielded power law relations and D_f values that were comparable to ones reported in the literature for other cold-set protein gels. However, our rheological data did not fit within one of the reported fractal

aggregate network models (*5, 6, 7, 8*). This is in line with De Kruif et al. (*9*), who observed that application of the scaling laws of Bremer and Shih et al. to heat-induced WPI gels did not result in consistent or realistic values. None of the models mentioned yields realistic scaling parameters when trying to combine the concentration dependence of both small (storage modulus (**Figure 6**)) and large deformation measurements(limit of linearity (**Figure 7**)). Also, Ikeda et al. (*10*) observed that for heat-induced WPI gels under certain conditions, the dependence of the limit of on the protein concentration is incorrectly predicted by fractal models.

A likely explanation for the fact that none of the known models consistently described our data could be that acid-induced aggregation indeed starts off as a random fractal aggregation, but at a certain length scale was frustrated or taken over by another mechanism. Recently, it has been proposed for heat- and solvent quality(ethanol)-induced globular protein gelation that, during gelation, both demixing by gelation and binary phase separation occurred (*27, 28, 29*). Indeed, in preliminary aggregation experiments with our system, where the aggregation of blocked and unmodified aggregates (protein concentration 0.5%) was monitored by small-angle light scattering, spinodal decomposition was observed (R. H. Tromp, personal communication). If indeed spinodal decomposition occurs, this will lead to locally higher concentrations of (unmodified) aggregates, allowing the formation of disulfide bonds in those concentrated areas, yielding an increase in aggregate size after gelation and resolubilization. The formation of larger disulfide-linked aggregates has indeed been demonstrated (*16*). This implies that the resulting spatial distribution of the disulfide links was not of a purely fractal nature. Although the microstructures of gels made from blocked or unmodified aggregates did not differ (length scale > 100 nm), the difference in the spatial distribution of larger disulfide-linked aggregates will certainly have affected the rheological properties and the applicability of the different fractal models.

Moreover, it was also observed that once a gel was formed, structural rearrangements probably occurred during aging of the gels. The occurrence of rearrangements was more pronounced for the blocked aggregates. These changes in microstructure can take place at a length scale smaller than 100 nm and could affect gel parameters such as the storage modulus and limit of linearity and therefore the applicability of the fractal models. It should be mentioned that this study was limited to the final gelation process and consequently covers only a limited protein con-

centration range. At lower concentration, the spinodal decomposition may be less pronounced, and during the initial heating step when no gel is formed, the rearrangements as discussed here will not occur. This may explain why, under these conditions, fractal scaling over many orders of magnitude of light-scattering intensities can be measured (*30*, *31*).

In conclusion, the rheological data support our hypothesis that the first stage of the cold-gelation process leading to a space-filling protein network is driven by physical interactions (*2*, *16*). The second stage is characterized by an increase in the gel stiffness and hardness by formation of additional covalent bonds between the structural elements of the gel. Analysis of the structural characteristics of acid-induced gels of both thiol-blocked and unmodified WPI aggregates yielded a fractal dimension (2.3 ± 0.1), which is in line with other comparable protein networks and suggests a reaction-limited aggregation mechanism (*25*). However, application of known fractal scaling equations to our rheological data yielded ambiguous results. Probably, gelation starts off as a fractal process but is frustrated by another mechanism at larger length scales (>100 nm). In addition, evidence was found for structural rearrangements at smaller length scales (<100 nm). These rearrangements were enhanced by the loss of the ability to form disulfide cross-links and will also disrupt the applicability of the concept of fractals on acid-induced cold-set gels of whey proteins.

ACKNOWLEDGMENT

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Acid-induced cold gelation of ovalbumin and whey protein isolate, a comparative study

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Acid-induced cold gelation of ovalbumin and whey protein isolate, a comparative study

Summary

The process of cold gelation of ovalbumin and the properties of the resulting cold-set gels were compared to those of whey protein isolate. For both protein preparations heating conditions were chosen such that most protein was organized in covalently-linked aggregates with comparable numbers of thiol groups exposed at their surfaces, but with clearly different shapes. During acid-induced gelation, the characteristic ordering caused by the repulsive character disappeared and was replaced by a random distribution. This process did not depend on aggregate characteristics and probably applies to any type of protein aggregate.

Covalent bonds are the main determinants of the gel hardness. The formation of additional disulfide bonds during gelation depended on the number and accessibility of thiol groups and disulfide bonds in the molecule and was found to differ significantly between the protein studied. However, upon blocking of the thiol groups long fibrillar structures of ovalbumin contribute significantly to gel hardness, demonstrating the importance of aggregate shape.

Introduction

The ability to gel is an important function of proteins in food systems. Proteins from different sources can produce gels which vary in textural properties (*1*). Most food protein gels are formed during heating and are therefore referred to as heat-induced gels. For relatively a small number of proteins, gelation at ambient temperature is reported (*2*). This so-called cold-gelation is a two-step process. In the first step, a solution of native proteins is heated and soluble aggregates are formed by heating at a pH distant from the iso-electric point and at low ionic strength. Upon cooling, the aggregates remain soluble and no gelation occurs. In the second step, gelation is induced at ambient temperature by reduction of the electrostatic repulsion (*3*), either by adding salt or by changing the pH towards the iso-electric point of the proteins. Gelation caused by lowering pH is called acid-induced gelation.

In the literature, several studies have reported cold gelation of whey protein isolate (WPI) (*1, 3, 4-19*). Most of these concerned the salt-induced type of cold gelation of WPI and only a few studies dealt with the acid-induced type (*3, 14, 15,17-19*). Previously (*3, 17-19*), we demonstrated that in the acid-induced cold gelation process, first a protein network is formed by physical interactions, which is subsequently stabilized by the formation of disulfide bonds. The formation of disulfide bonds took place in spite of the acidified conditions and will likely also occur under more favorable conditions in the salt-induced type of cold gelation of WPI. The formation of disulfide bonds was shown to be of great importance for the mechanical properties of cold-set WPI gels and for their physical stability (syneresis) (*3, 17-19*).

Whereas the cold gelation of WPI was intensively investigated, far less is known about the cold gelation of proteins in general. Therefore, in this study the cold gelation process of ovalbumin was compared with that of WPI. In contrast to WPI or β-lactoglobulin (β-lg) that both form aggregates with a curved strand-like morphology (*18, 20*), ovalbumin can form soluble fibrillar aggregates (*21*) under the conditions applied in the cold gelation process.

Like β-lg, ovalbumin is a globular protein with a net negative charge at neutral pH (iso-electric point 4.7). It has a molecular mass of 46 kDa and contains six cysteine residues, two of which form a cystine. As for β-lg, the thiol groups are buried in the three-dimensional structure of the native protein, but are exposed upon denaturation of the protein molecule. They play an important role in the heat-induced aggregation of ovalbumin (*22, 23*).

In contrast to heat-induced gelation, in which aggregation and gelation occur at the same time, in cold gelation these two processes occur separately. Here we took advantage of the fact that in the first step stable soluble aggregates were formed, of which the properties could be controlled before gelation was induced. Covalently-linked aggregates of ovalbumin and WPI were prepared, which were comparable in terms of their hydrodynamic diameter, electrophoretic mobility, and number of thiol groups, but were clearly differently shaped. Results on the cold gelation of ovalbumin are discussed and compared to the cold-gelation of WPI.

Material and methods

Reagents and chemicals. Glucono-δ-lactone (GDL), 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), sodium dodecylsulphate (SDS), Rhodamine B and *N*-ethylmaleimide (NEM) were obtained from Sigma Chemicals (St. Louis, MO, USA). 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 a kind gift from Davisco Foods International Inc. (Le Sueur, MN, USA). The WPI consisted (based on dry weight) of β-lg (74%), α-lactalbumin (α-lac)(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%) (*24*). Ovalbumin was purified from fresh chicken egg white based on the procedure of Vachier et al. (*25*).

Preparation of aggregates. WPI aggregates were prepared at protein concentrations of 3 and 9% (w/w) as described elsewhere (*18*). Ovalbumin was dissolved in double-distilled water at ambient temperature at a protein concentration of 2 and 5% (w/w) (*26*). Ovalbumin aggregates were prepared by heating the ovalbumin solutions in a water bath for 22 h at 78°C.

After heating, the dispersions of aggregates were rapidly cooled to ambient temperature in 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 (*27*). The dispersions of aggregates were stored at 4°C until use (typically, within a few days) and diluted to 2% (w/w) with double-distilled water just before the start of the gelation experiments.

Blocking and determination of reactive thiol groups. The number of accessible thiol groups at the surface of the aggregates before and after chemical blocking was determined using DTNB, also known as Ellman's reagent (*28*) as described elsewhere (*17*). The assay was performed in the absence of urea and SDS, to avoid measurement of non-surface thiols. NEM was added to a final concentration of 2 mM to block the remaining surface thiol groups (*17*). The effectiveness of this treatment was also checked for ovalbumin by the use of the Ellman's reagens.

SDS-agarose electrophoresis. SDS-agarose gel electrophoresis (0.4 % (w/w) agarose) was performed as described before (*17*) to determine the differences in molecular size of the differently treated protein aggregates. Aggregates (in solution or gel) were dissolved in an SDS-containing buffer (5% SDS, pH 7.0) to a final protein concentration of 0.5% (w/w). Staining was done with Phastgel Blue R.

GDL-induced gelation. GDL was added as a powder to the dispersions of aggregates (concentration of protein 2% (w/w)) to induce cold gelation at ambient temperature. To observe gelation of the aggregates around their iso-electric points, it was required to add 0.15% (w/w) and 0.16% (w/w) of GDL to WPI and ovalbumin solutions, respectively. The pH was monitored simultaneously in samples placed in a water bath kept at 25°C or determined after 21 h of incubation at ambient temperature. The point of gelation was determined by means of small deformation measurements.

Turbidity measurements. Turbidity measurements were made at 25°C and at a protein concentration of 2% (w/w) on a Cary 1E UV-Vis spectrophotometer (Varian) equipped with a temperature controller. The turbidity was measured at 500 nm with time using cuvettes with a path length of 1 mm.

Scattering techniques. Small angle X-ray scattering (SAXS) measurements were made at the Dutch-Belgian beam-line (DUBBLE) at the European Synchrotron Radiation Facility at Grenoble (France). The energy of the beam was 12.2 keV (wavelength $\lambda = 1.015$ Å) and 8 keV (wavelength $\lambda = 1.55$ Å), the detector was a two-dimensional (512 x 512 pixels) gas-filled detector placed at 8 m distance. The scattering wave vector was between 0.1 and 1.0 nm^{-1} (corresponding to a length scale between 6.3 and 62.8 nm in real space). The temperature of the samples was kept constant at 25°C.

Small angle neutron scattering was performed as described by Tuinier et al (*24*). The scattering wave vector was between 0.015 and 0.75 nm^{-1} (corresponding to a length scale between 8.4 and 42.4 nm in real space).

Small angle light scattering (SALS) experiments were done on a custom-built set-up (*29*). The scattering wave vector was detected between 0.7 and 3.0 μm^{-1} (corresponding to a length scale between 2 and 9 μm in real space). The protein concentration of the samples was 2 % (w/w) in all cases; the temperature was kept at 25°C. The scattering pattern of the samples was recorded as a function of time during 20 h after the addition of GDL.

Confocal microscopy. Imaging was performed as described elsewhere (*17*). The protein gels were stained by applying 2 μl of an aqueous solution of 0.05% Rhodamine B. Confocal images were made of the cold-set WPI gels acidified for 24 h. Fast Fourier transform (FFT) spectra were made from these images, with a range between 0.81 and 160 μm^{-1} (corresponding to a length scale between 39 nm and 7.78 μm in real space)

Rheological measurements. Small-amplitude oscillatory measurements were made with a Carri-Med CLS^2 500 rheometer (TA Instruments, Leatherhead, UK) using a sand-blasted (roughened) conical concentric cylinder measuring unit (inner radius 8.60 mm, outer radius 9.33 mm). Immediately after the addition of GDL, samples were brought into the concentric cylinder and covered with a thin layer of paraffin oil to prevent evaporation. All experiments were done in oscillation mode at a frequency of 1 rads^{-1} (0.159 Hz) and an applied strain of 1%, which is within the linear region. The formation of a gel network was monitored by the development of the storage modulus (G') and loss modulus (G") during 21 h at 25°C. The maximum linear strain was determined by a strain sweep experiment.

Gel hardness was determined by deformation under a compression force approximately 21 h after the addition of GDL, as described elsewhere (*17*) by means of a texture analyzer (type TA-XT2, Stable Micro Systems Ltd., Godalming, England) equipped with a wire mesh-device.

Cryo-TEM of dispersions of aggregates. Cryo-transmission electron microscopy was done as described elsewhere (*26*), using a Philips CM12 transmission electron microscope operating at 80 kV. Images were recorded digitally by a Gatan 791 CCD camera using the Digital Micrograph software package.

TEM of acid-induced cold-set gels. Samples of cold-set gels were fixed in 2.5 % glutaraldehyde for 1 h and stored in 0.25% glutaraldehyde at 4°C for 48 h. The fixed samples were rinsed in distilled water and dehydrated at ambient temperature in a graded series of acetone/water (70%, 80%, 96%) for 15 min each, followed by 100% acetone (2x30 minutes). An epoxy resin, EMBED 812, was infiltrated using graded mixtures of EMBED 812 and acetone in three steps of 1 h each. Final infiltration was done overnight in 100% epoxy resin. The samples were embedded in BEEM capsules and subsequently polymerized at 60°C for 48 h. After polymerization, thin sections were cut using a Leica Ultracut FCS ultramicrotome and collected on 100 mesh-Formvar-coated copper grids. The protein on the grids were stained for 1 min with Reynold's lead citrate followed by 15 s of saturated uranyl acetate in distilled water. The grids were imaged using a Philips CM12 transmission electron microscope operating at 80 kV accelerating voltage.

Results and discussion

Preparation of disulfide cross-linked ovalbumin aggregates. Protein aggregates of ovalbumin were prepared by heating solutions of native proteins at pH 7 and at low ionic strength. For WPI this resulted in dispersions of protein aggregates consisting of covalently cross-linked protein monomers (*17, 30*). Agarose gel electrophoresis in the presence of the denaturant SDS, was used to demonstrate the formation of aggregates of covalently linked ovalbumin monomers under comparable conditions (**Figure 1**).
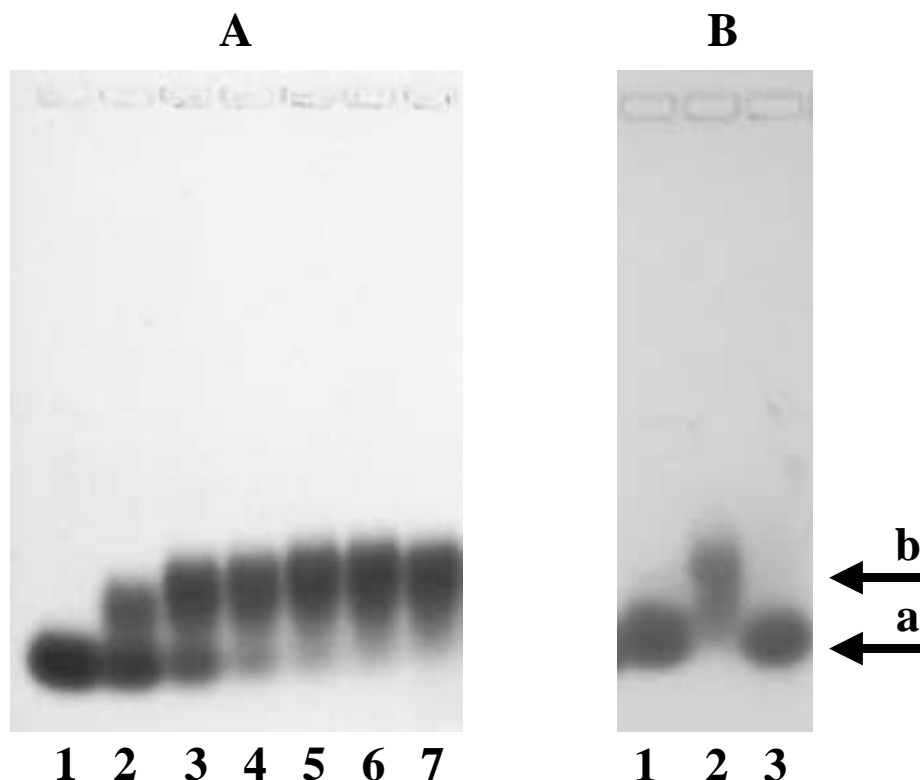
Figure 1: (A) SDS agarose gel electrophoresis of heated and unheated ovalbumin solutions (3 %, 75°C). Lane 1, unheated ovalbumin, lanes 2-7 ovalbumin heated for 30, 300 1026, 2683, 5000 min and for 8 days. (B) SDS agarose gel electrophoresis of ovalbumin aggregates with and without the addition of DTT. Lane 1: unheated ovalbumin (monomers), lane 2: ovalbumin aggregates (5 % protein 22 h 78°C) without DTT, lane 3: ovalbumin aggregates with DTT.
Arrows a: protein monomers, arrow b protein aggregates.

Figure 1A shows that upon prolonged heating of a 3% (w/w) ovalbumin solution at 75°C, aggregates were formed that did not dissociate in the presence of SDS. Similar to WPI (*17*), the migration velocity of the protein band caused by the ovalbumin aggregates becomes similar to that of the ovalbumin monomers, after addition of DTT (**Figure 1B**). Since DTT is known to disrupt disulfide bonds, the electrophoresis results strongly indicated that, like the WPI aggregates, ovalbumin aggregates consisted of disulfide-linked ovalbumin monomers.
The kinetics of denaturation and formation of internally disulfide cross-linked aggregates of ovalbumin were clearly different from that of WPI, where denaturation and formation of disulfide cross-linked aggregates under the applied conditions is reported to go hand in hand (*30*). **Figure 1A** clearly shows that even after heating for 300 min the major part of the denatured ovalbumin has not been converted into aggregates consisting of disulfide cross-linked monomers, whereas after 180 min of heating at 75°C more than 99% of the native ovalbumin molecules is denatured, insoluble at

pH 4.6, and physically aggregated (unpublished results). Prolonged heating, up to 17 h, was required to form the covalent disulfide bonds in physically aggregated denatured ovalbumin molecules. Kitabatake et al. (*22*) also found that in the early stage of aggregation non-covalent bonding played a major role instead of covalent disulfide bonding. Hence, for denatured ovalbumin, physical aggregation of monomers preceded the formation of internal disulfide bonds within these aggregates.

With the aid of SDS-agarose electrophoresis, we determined the time needed at a temperature of 78°C to form aggregates consisting of covalently cross-linked monomers in which more than 90% of the ovalbumin participated was determined. Independent of the protein concentration this time was 22 h (**Table 1**).

Table 1: Characteristics of WPI and ovalbumin aggregates.

Sample[a]	Heating conditions	Number of thiol groups (mM)[b]	Hydro-dynamic radius (nm)[c]	Contour length determined by cryo-TEM (nm)	Percentage denaturation[d] (%)
3% WPI	24 h 68.5^{o}C	0.18	15	20-60	>95
9% WPI	2 h 68.5^{o}C	0.34	38	40-100	>95
2% ova	22 h 78°C	0.26	12	30-150	>99
5% ova	22 h 78°C	0.40	35	400-700	>99

a   Protein concentration given is the concentration at heating (w/w).

b   Determined at pH 7 with Ellman's assay (Ellman, 1959). The number of thiol groups was determined using ϵ_{412nm}= 13,600 M^{-1} cm^{-1} for 2-nitro-5-mercaptobenzoic acid and expressed as the concentration of thiol groups (mM) in a 2% (w/w) dispersion of protein aggregates. Measurements were done in duplicate with an experimental error lower than 10%.

c   The hydrodynamic radii were determined by means of dynamic light scattering, assuming that the aggregates have a roughly spherical shape (Alting et al., 2003; Weijers et al., 2002).

d   According to Alting et al. (2003) and Weijers et al. (2003). The concentration 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 concentration of native proteins in the unheated sample was considered as 100% (0% denaturation). Measurements were performed at least in duplicate with an experimental error lower than 5%.

Characterization of protein aggregates.

Size. In the present study, protein solutions were heated at two concentrations, one far below and one close to the reported gelation concentration (*9, 26, 31*). As reported elsewhere, the heat treatment of protein solutions at increasing initial protein concentrations resulted in protein aggregates with increasing hydrodynamic radii as determined by dynamic light scattering (**Table 1**)(*15, 18, 21, 26, 27*). For both proteins the electrophoretic mobility depended on the protein concentration at heating and decreased with increasing protein concentration (not shown). Some caution is justified, because the apparent radii of the aggregates in solution were calculated from a standard cumulant fit of the auto-correlation function of the scattered intensity, assuming that the aggregates have a roughly spherical shape. Hence, these results are only indicative. Indeed, cryo-TEM observations demonstrated that the aggregates prepared did not have a spherical shape (see below).

Shape. Microstructural analysis by means of cryo-TEM showed (**Figure 2; Table 1**) that after heat treatment at low ionic strength, a pH around 7 and dependent on the protein concentration, aggregates were formed differing in shape and size, curved strand-like and long fibrillar aggregates for WPI (*18, 20*) and ovalbumin (*21, 26*), respectively.
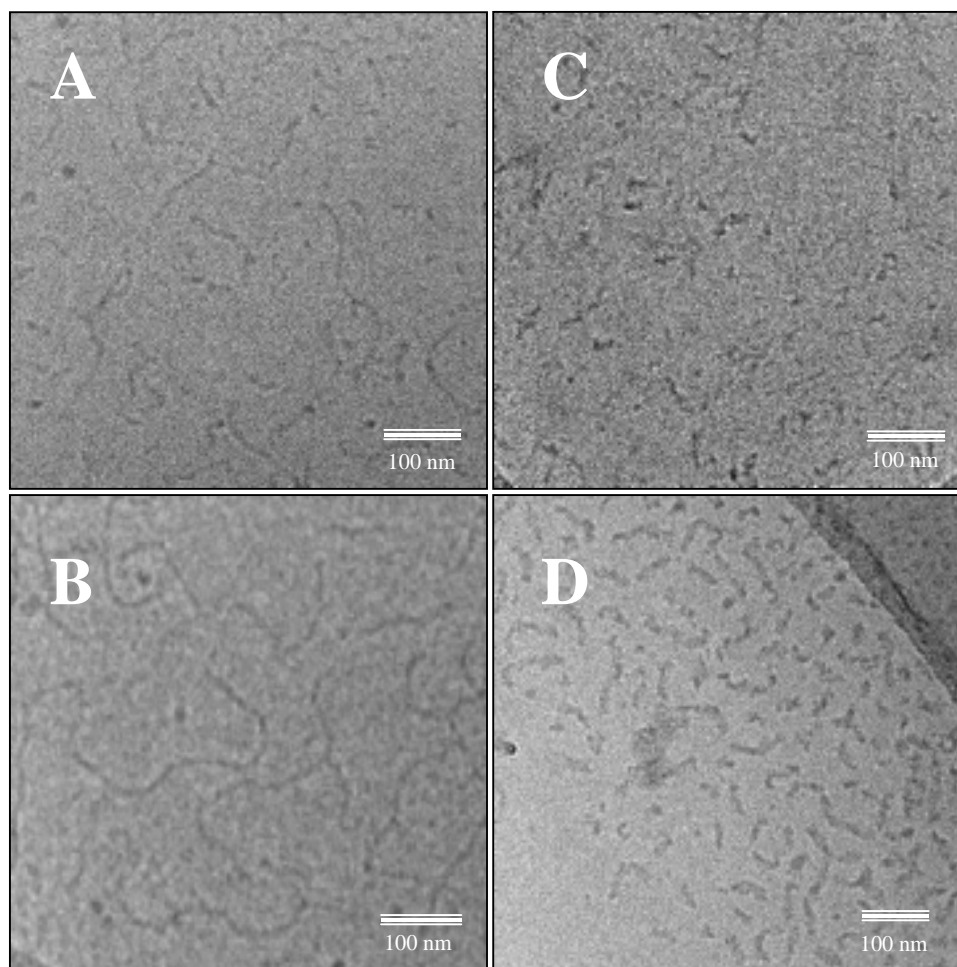
Figure 2: Cry-TEM micrographs of dispersions of aggregates heated at different protein concentrations (w/w). (A) 2.5% ovalbumin, 22 h 78°C; (B) 5% ovalbumin, 22 h 78°C; (C) 3% WPI, 24 h 68.5°C and (D) 9% WPI, 2 h 68.5°C.

Under the applied conditions, both proteins had a net negative surface charge and formation of soluble linear aggregates is therefore promoted (*32*). Note that the particle sizes as observed by cryo-TEM appeared to be significantly larger than those observed with dynamic light scattering (DLS), which can probably be explained by the elongated shape of the aggregates.

Number of exposed thiol groups. **Table 1** shows that at the point in time that 95-99% of the protein was aggregated into covalently linked monomers, a lower number of thiol groups was detectable for aggregates prepared at a lower initial protein concentration. For WPI this was explained by differences in the occurrence of oxidation or degradation reactions (*18, 33, 34*) caused by a difference in heating time to reach 95% aggregation. The results of ovalbumin heated at different protein concentrations cannot be fully explained in this way, as these were heated for the same period of time. The blocking of the thiol groups by means of a treatment with NEM had no effect on the size as determined with both DLS and agarose electrophoresis for both the WPI and ovalbumin aggregates.

Acid-induced cold gelation. Before gelation was induced by the addition of GDL, dispersions of aggregates prepared at various protein concentration were diluted to a protein concentration of 2% (w/w). In water, GDL slowly hydrolyzes, yielding gluconic acid, which caused a gradual decrease of the pH and induces gelation of the dispersions of aggregates towards their iso-

electric points. The resulting properties of the final cold-set ovalbumin gels such as the appearance and the large deformation mechanical properties, clearly differed from cold-set WPI gels.

Appearance of cold-set gels. The ovalbumin gel prepared from a dispersion heated at a protein concentration of 2% is translucent and the gel prepared from a dispersion prepared at 5% (w/w) is transparent (**Figure 3**).
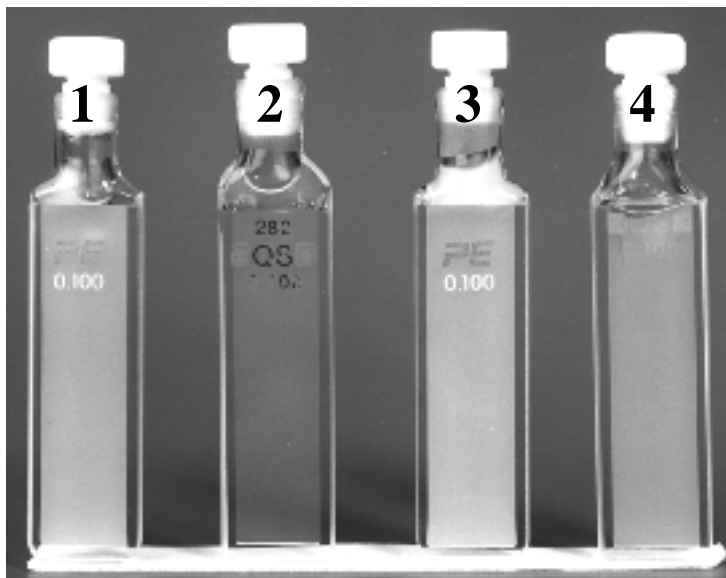

Figure 3: Appearance of cold-set gels (2% (w/w) protein) of ovalbumin and WPI as observed in cuvettes with at a path length of 1 mm.
1: WPI, initial protein concentration 9% (w/w); 2: Ovalbumin, initial protein concentration 5% (w/w); 3: WPI, initial protein concentration 3% (w/w); 4: Ovalbumin, initial protein concentration 2% (w/w).

On the other hand, both the WPI gels, prepared from dispersions initially heated at protein concentrations of 3% and 9% (w/w), were turbid. (see also **Table 2**, A_{500nm} after 21h). Kitabatake et al. (*22*) also reported the formation of transparent ovalbumin gels using a different gelation method. Visually, treatment with NEM had no influence on the appearance, although small but reproducible differences in turbidity could be determined for WPI (**Table 2**), which is discussed elsewhere (*19*).

Large deformation properties. Figure 4 shows the force-distance curves of cold-set gels of both ovalbumin (**Figure 4A**) and WPI (**Figure 4B**) determined by gel penetration measurements. For cold-set gels of ovalbumin, we observed a clear effect of the protein concentration at heating on the gel hardness (**Figure 4A**). More force (approximately 6 times) had to be applied to penetrate gels prepared from dispersions heated at a protein concentration of 5% (w/w) than gels prepared from dispersions heated at 2% (w/w). Surprisingly, no effect of the thiol-blocking agent on gel hardness was observed. This suggests that instead of the ability to form disulfide bonds, other aggregate characteristics, such as the shape will contribute to these differences.

As reported previously (*17, 18*), 2% (w/w) cold-set WPI gels of dispersions initially heated at 9% (w/w) protein were approximately 10 times stronger than gels prepared from dispersions initially heated at 3% (w/w) protein (**Figure 4B**). There, we concluded that the hardness of cold-set WPI gels, in contrast to ovalbumin, is determined by the number of thiol groups rather than by the size of the aggregates or other structural features. **Figure 4B** shows that indeed only a minor effect of the difference in hydrodynamic diameter (**Table 1**) on the gel hardness remained, if the thiol groups present on the surface of the WPI aggregates were chemically blocked.
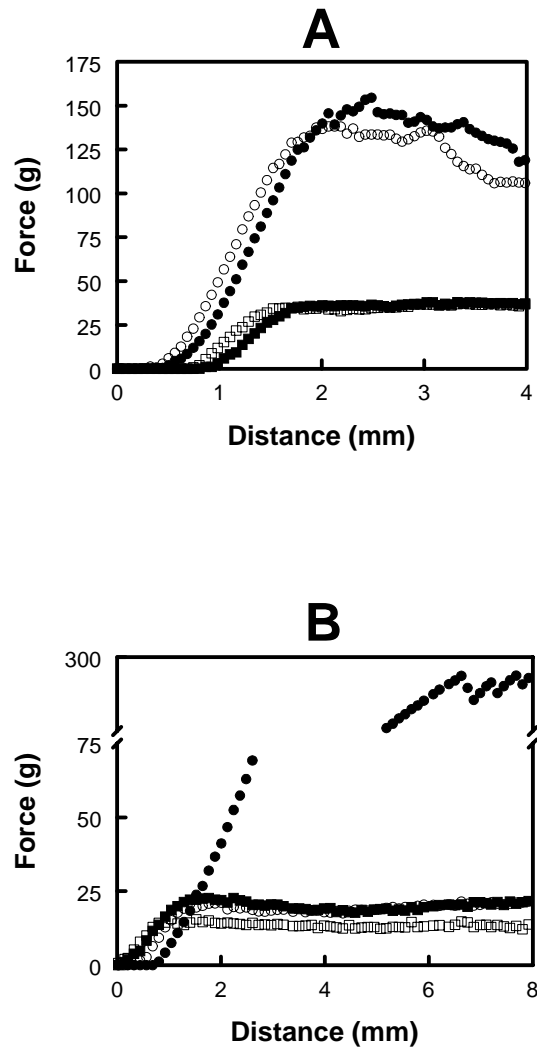
Figure 4: Effect of thiol-blocking on the large deformation properties of acid-induced cold-set gels of ovalbumin (A) and of WPI (B) at a protein concentration of 2% (w/w) after 24 h of acidification. The circles represent the higher initial protein concentrations at heating, 5% (w/w) and 9% (w/w) for ovalbumin and WPI, respectively. The squares represent the low initial protein concentrations at heating, 2% (w/w) and 3% (w/w), for ovalbumin and WPI, respectively. The closed symbols represent the untreated aggregates, the open symbols the NEM-treated aggregates.
Note that there is break in the y-ordinate in Figure 4B.

To determine the effect of structural properties (size or shape) of protein aggregates on gel hardness, cold-set gels prepared from aggregates treated with a thiol-blocker were compared. The effect of the initial protein concentration at heating on gel hardness was much larger for ovalbumin (approximately 600%) than for WPI (approximately 20%). This significant difference cannot be merely explained by differences in the number of junctions, which directly relate to the value of G'. The effects of the initial protein concentration on small deformation properties were much smaller (see below). The results can possibly be explained by the formation of entanglements in the case of significantly longer fibrils (40-60 versus 500 nm) in the case of ovalbumin.
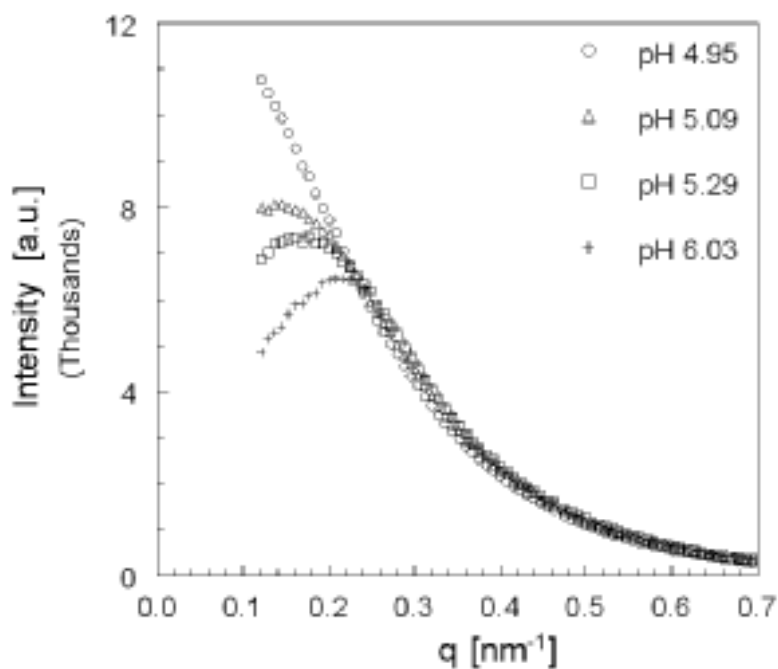
Figure 5: Evolution of the SAXS pattern of a 4% (w/w) solution of ovalbumin aggregates during acidification.

Structural properties. In **Figure 5**, the SAXS patterns are shown for the acid-induced cold gelation of ovalbumin. Before acidification, a clear peak was observed, corresponding to an ordering of aggregates with a typical inter-particle distance of approximately 26 nm, that shifted towards lower q-values upon acidification. Simultaneously, broadening of the peak was observed and finally the peak disappeared, which indicated that the dominant length scale (inter-particle distance), present before acidification, disappeared. Comparable results were found by the addition of an increasing amount of sodium chloride (results not shown).

Furthermore, no significant differences between the SAXS patterns of untreated and NEM-treated aggregates were observed during the acidification. For WPI the same results were obtained, but at a significantly higher protein concentration (higher than 6% (w/w)) than for ovalbumin (2% (w/w)), by the use of both SAXS and SANS. At a protein concentration of 9%, typical inter-particle distances of approximately 25 nm and 26 nm were observed for WPI-aggregates by the use of SANS and SAXS, respectively.

The SAXS measurements also showed that after a gel is formed at a certain pH (see also **Table 2**), the structure continued to change upon lowering the pH. This suggests that once a gel was formed, structural rearrangements still took place. These observations were confirmed by small deformation and turbidity measurements, that continued to increase even after the final pH was reached (see below).

SALS measurements of ovalbumin aggregates in solution showed no dominant length scale. During acid-induced gelation an increase in scattering intensity, which was not q-dependent in the wave vector range measured, was observed, but no dominant length scale was observed. Similar results were obtained for WPI, which were consistent with the confocal microscopy results for WPI described below. The protein structures in the transparent ovalbumin gels could not be visualized probably because these structures were smaller than the resolution of the microscope.

Confocal images were made of the cold-set WPI gels 24 h after GDL addition. No difference was observed in the 2D Fourier spectra of confocal images of gels formed from aggregates with or without thiol-blocking treatment. Furthermore, no dominant length scale was

observed in this range; therefore we concluded that both the ovalbumin and WPI cold-set gels were homogeneous at the length scales probed (over a range of 6.3 nm to 9 μm in real space).

Small deformation and scattering properties. Figure 6 shows representative results of the development with time of the storage modulus (G'), the turbidity (absorbance at 500 nm) and the pH during acidification of dispersions of both ovalbumin (**Figure 6A**) and WPI (**Figure 6B**) aggregates, with and without NEM treatment. In **Table 2**, the results are summarized in six parameters, namely the G' after 21 h, the turbidity after 21 h, point in time of the initial increase in G' (gelation point), point in time of the initial increase in turbidity, the pH at the gelation point and the maximum linear strain.

Since the acidification curves (pH versus time) of the two proteins were almost identical, results were compared on a time scale instead of a pH scale. There seems to be a relation between the length of the protein aggregates and the point of gelation, defined as the initial increase in G'. Long fibrils (ovalbumin, initially heated at 5% (w/w)) showed the earliest point of gelation in time and the shortest linear aggregates (WPI, initially heated at 3% (w/w)) showed the latest point of gelation at the same protein concentration. NEM treatment had no significant effect on the gelation point in all cases.
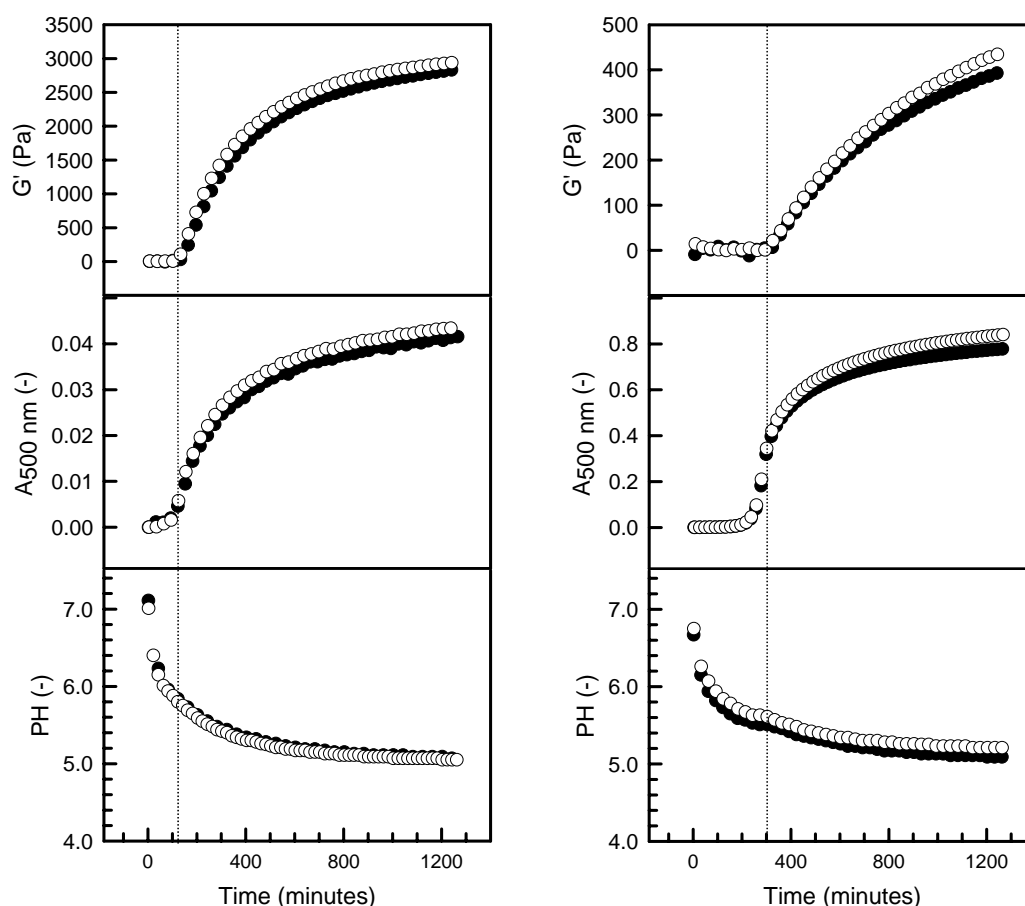

Figure 6: Development of the storage modulus (G'), the turbidity (expressed as $A_{500\ nm}$) and the pH with time for WPI, initially heated at 3% (w/w) (left panels) and for ovalbumin, initially heated at 5% (w/w) (right panels) after the addition of GDL. The closed symbols represent the untreated aggregates, the open symbols the NEM-treated aggregates. Note that the y-ordinate values are different for the left and right panels.

Table 2: Rheological and scattering characteristics of acid-induced gelation of ovalbumin and WPI.

Sample	G' (21h) (Pa)	$A_{500 nm}$ (21 h)	Initial increase in G' (Gelation point) (min)	Initial increase in $A_{500 nm}$ (min)	pH at gelation	Maximum linear strain (%)
9% WPI	680	0.57	235	200	5.48	55
9% WPI+NEM	663	0.70	240	230	5.57	14
3% WPI	399	0.80	323	263	5.52	11
3% WPI+NEM	435	0.86	300	246	5.59	8
5% ovalbumin	2980	0.045	130	114	5.82	22
5% ovalbumin + NEM	2960	0.044	130	115	5.78	17
2% ovalbumin	1093	0.23	185	169	5.65	11
2% ovalbumin + NEM	1050	0.22	210	195	5.70	11

Values given are average values. Measurements were done at least in duplicate with an experimental error lower than 10% and lower than 5% for WPI and ovalbumin, respectively.

The G' value reached 21 h after the addition of GDL increases from about 400 Pa for the shortest linear aggregates to approximately 3000 Pa for the longest linear aggregates. G' and G" developed concurrently with time, G' being dominant over the whole time scale measured. Tan δ (G"/G') was the same (0.13-0.15) for all systems. For gel systems, this relatively high value indicates that the visco-elastic gel systems still contained a significant viscous part (G"). NEM treatment of the aggregates had only a minor effect on the evolvement of the storage modulus (G').
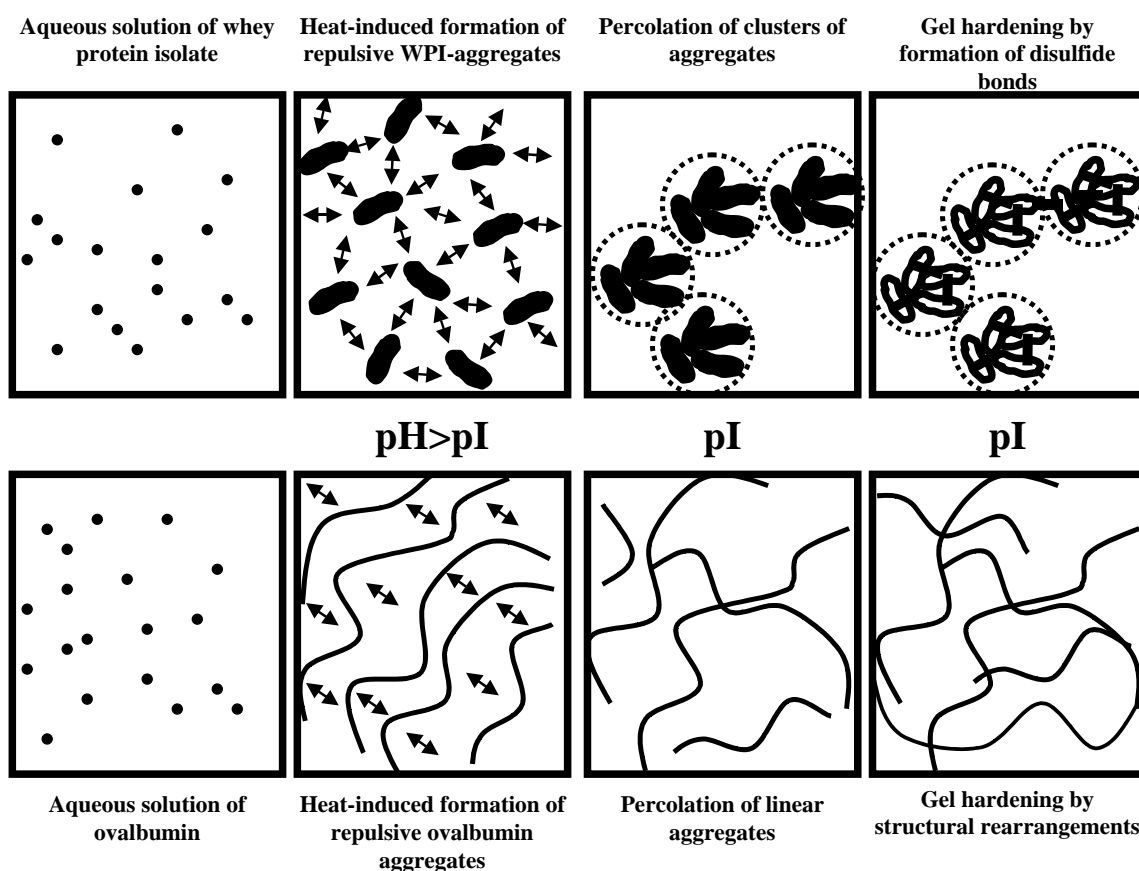
Figure 7: Schematic representation of the acid-induced cold gelation of small (upper panels) and long (lower panels) linear protein aggregates.

To explain the formation of disulfide bonds and the effect of that on the large deformation properties of acid-induced cold-set WPI gels, we previously (*3, 17-19*) postulated that, first a protein network was formed by physical interactions, which was subsequently stabilized by the formation of disulfide bonds. Here we extended this model (**Figure 7**) and explained the large differences in G' and turbidity of the various protein systems by differences in overlap concentration.

Cold gelation was induced by reduction of electrostatic repulsion between aggregates (*3*). In the case of relatively short linear aggregates (**Figure 7** upper panels), the aggregates were too small to percolate directly. In this case, the aggregates first became organized in clusters, which were subsequently able to form a space-filling network. This was supported by the observation that shorter linear aggregates (WPI 3% (w/w)), showed an increase in turbidity, indicating an increase in aggregate size, before a gel was formed. Only the number of junctions between the clusters will mainly determine the small deformation properties (in linear region) of the final gels. In the case of relatively long linear aggregates (**Figure 7** lower panels), formation of a space-filling network occurred immediately after reduction of electrostatic repulsion, resulting in shorter gelation times. In contrast to the short linear WPI aggregates, SAXS measurements showed that the long ovalbumin aggregates maintained a typical inter-particle distance at this protein concentration caused by electrostatic repulsion. By the reduction of the repulsion, the aggregates were able to directly form a space-filling network. Most of the junctions between the individual fibrils will contribute to the small deformation properties, which explain the higher G' values found for this type of protein gels.

The maximum linear strain for WPI initially heated at 9% (**Table 2**) was significantly larger than for the other samples. Reduction of the number of thiol groups, either by chemical blocking or by heating at relatively low protein concentration resulted in a clear reduction of the maximum linear strain. For ovalbumin, the effect of NEM was not significant. However, the maximum linear strain did vary with the size of the ovalbumin aggregates. These results are in good agreement with those obtained from the penetration experiments.

TEM of acid-induced protein gels. **Figure 8** shows TEM micrographs of cold-set gels prepared from protein solutions initially heated at different concentrations. The TEM micrographs confirmed our postulated model (**Figure 7**). WPI gels prepared from an initial protein concentration of 3% (**Figure 8c**) clearly shows clusters of small aggregates, this is less pronounced for WPI gels prepared from an initial protein concentration of 9%, which shows less dense structures. As expected, gels prepared from ovalbumin fibrils show a more homogeneous structure (**Figures 8b**).The effect of NEM on gel structure was determined for WPI gels, and showed no difference with the control. Note that these micrographs were taken from cross-sections through the gel and not from a thin film as in the cryo-TEM measurements, therefore long fibrillar structures as observed in **Figure 2** cannot be observed.
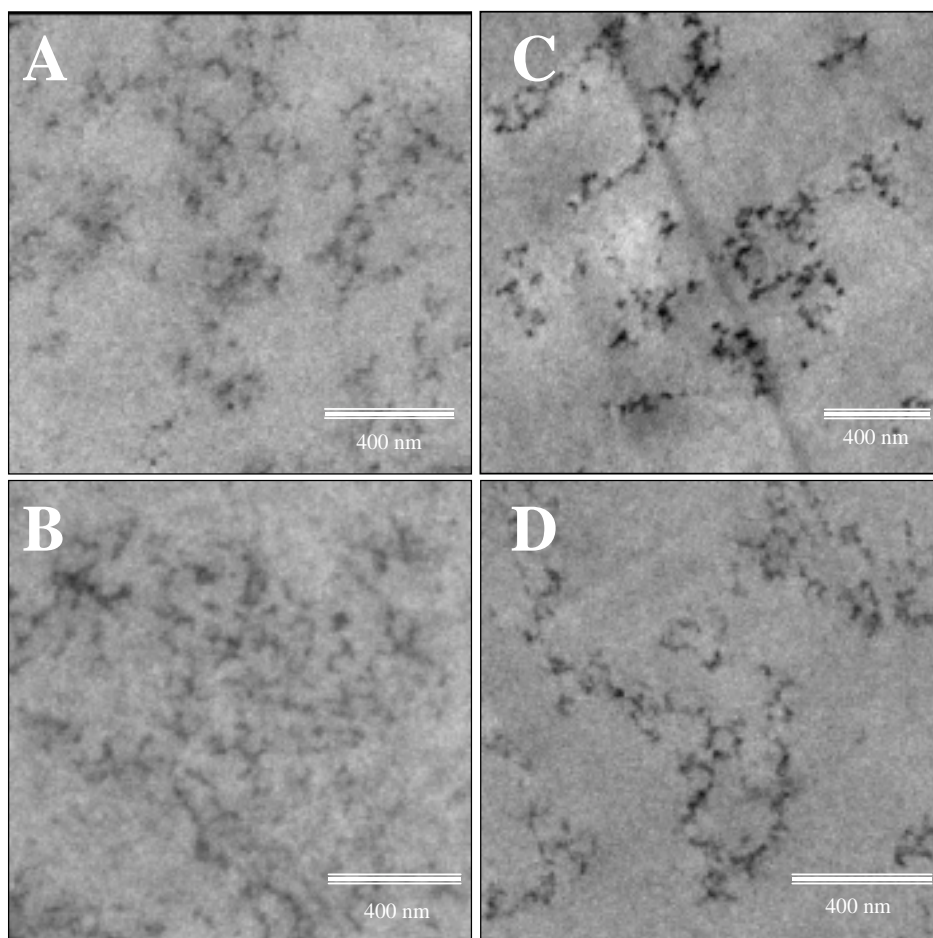
Figure 8: TEM micrographs of 2% (w/w) cold-set protein gels of ovalbumin and WPI initially heated at different protein concentrations (w/w). Ovalbumin, 2% (A), ovalbumin, 5% (B), WPI, 3% (C) and WPI, 9% (D).

Determination of disulfide cross-linking in acid-induced gels. Finally, to observe differences in the size of the protein structures before and after acid-induced gelation, SDS-agarose electrophoresis was applied (**Figure 9**). The acid-induced ovalbumin gels dissolved more easily in the SDS-containing electrophoresis buffer than the WPI gels. Unexpectedly, but in agreement with the rheological results, electrophoresis of cold-set gels of ovalbumin did not reveal changes in electrophoretic mobility after gelation (**Figure 9**; lanes 5-8). The results of the electrophoretic analysis clearly demonstrated that no disulfide cross-linked structures were formed during the cold gelation of ovalbumin aggregates, although these contained reactive thiol groups.

As reported previously for cold-set gels of WPI (*17, 18*), disulfide cross-linked protein structures were formed during gelation in the absence of the thiol-blocker NEM. The structures had a clearly decreased electrophoretic mobility (increased size) compared to the electrophoretic mobility of the initial aggregates (before gelation) (**Figure 9**; lanes 1 and 3). The differences in electrophoretic mobility observed between gels prepared from WPI aggregates heated at initial protein concentrations of 3% and 9% were explained by the difference in the number of the thiol groups and therefore in a difference in ability to form disulfide cross-links (*18*). In the presence of NEM, no decrease was observed in electrophoretic mobility (lanes 2 and 4), and the mobility was identical to that before gelation.
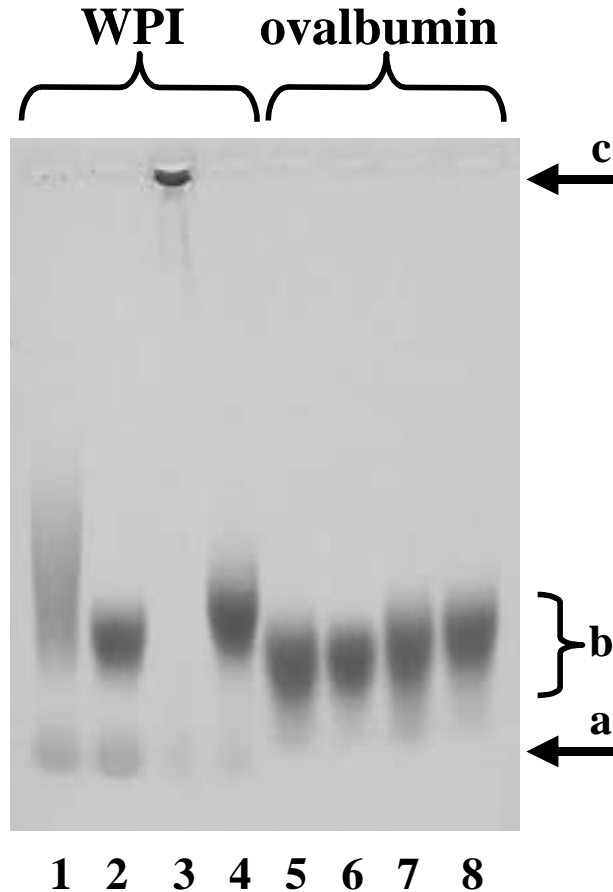
Figure 9: SDS agarose gel electrophoresis of, in SDS-containing buffer, redissolved cold-set gels of untreated and NEM-treated WPI (lanes 1-4) and ovalbumin (lanes 5-8).

Lane 1: 3% untreated WPI, lane 2: 3% NEM-treated WPI. Lane 3: 9% untreated WPI, lane 4: 9% NEM-treated WPI, lane 5: 2% untreated ovalbumin, lane 6: 2% NEM-treated ovalbumin, lane 7: 5% untreated ovalbumin and lane 8: 5% NEM-treated ovalbumin.

The protein concentrations given are the concentrations at heating (w/w). Arrow a: protein monomers, arrow b: protein aggregates, arrow c: protein structures that are unable to enter the agarose matrix.

Formation of disulfide bonds during the acid-induced gelation is of crucial importance for the properties of cold-set WPI gels (*17*). However, the results of large deformation experiments and the SDS-agarose electrophoresis presented here clearly show that during the acid-induced cold gelation of ovalbumin no additional disulfide bonds were formed, although such bonds did form during the preparation of the ovalbumin aggregates. An explanation might be found in the accessibility of the disulfide bond in ovalbumin. Different ways for the formation or reduction of disulfide bonds are known, but the one that normally takes place in the aggregation of proteins is the thiol-disulfide exchange reaction (18, 35, 36, 37, 38). As measured by the use of the Ellman's reagent, the thiol groups of the cysteine-residues were accessible for reaction after the heat-induced preparation of ovalbumin aggregates. In contrast, the accessibility of the disulfide bond can be different at conditions during heating (78°C and pH 7.2) compared to the conditions during gelation (ambient temperature and pH 5), due to (reversible) conformational changes of the ovalbumin molecule. The decreased accessibility of the disulfide bond will prohibit disulfide cross-linking during gelation. Legowo et al. (*39*) observed that the addition of α-lac, a protein containing four disulfide bonds, significantly increased the hardness of heat-set ovalbumin gels. This also confirms

that the thiol group of ovalbumin is available for reaction and that the disulfide bonds in α-lac are accessible for reaction with the heat-exposed thiol groups of ovalbumin, in contrast to the one in ovalbumin. Indeed, Legowa et al. (*39*) demonstrated the contribution of the Cys6-Cys120 disulfide bond of α-lac in the interaction between α-lac and ovalbumin.

Note that WPI is mixture of proteins. It mainly consists β-lg, but the second major protein is α-lac. Although formation of disulfide bonds was observed in acid-induced cold-set gels of pure β-lg (*3*), it was reported that WPI yields stronger gels after induction by high-pressure treatment and that this also caused by the incorporation of α-lac in the protein network (*40*). The same effect was observed in heat-induced gels of β-lg (*41, 42, 43*).

Conclusions

For both protein preparations, ovalbumin and WPI, repulsive aggregates were made consisting of disulfide cross-linked monomers. Both type of aggregates possessed exposed thiol groups at their surface (**Figure 7**). At pH 7, the aggregates of both WPI and ovalbumin had a net negative charge and typical inter-particle distances in the nm range were observed using different scattering techniques. This distance scaled with the protein concentration. If the net charge of the aggregates approached neutrality the characteristic length scale observed before gelation disappeared and over a wide range of length scales (nm to μm) no other characteristic length scale could be observed. Cold-set gel were formed by reduction of the electrostatic repulsion (*18*). Therefore we concluded that during this process the protein system changed from an ordered into a randomly organized structure. Since formation of the microstructure, as a result of the reduction of electrostatic repulsion, did not depend on aggregate characteristics, it can be defined as a non-protein-specific mechanism and therefore this mechanism will apply to any type of protein aggregate that is acidified towards its iso-electric point.

Differences in size and shape of the protein aggregates were found to influence small deformation properties of protein gels. Longer aggregates have a lower overlap concentration, which causes gelation to commence earlier and results in more effective junctions, yielding higher G' values.

Covalent bonds are the main determinants of the gel hardness. The formation of additional disulfide bonds after gelation not only depends on the number of thiol groups, but also on the number and accessibility of disulfide bonds in the molecule and may differ significantly from one protein to another. However, in the absence of covalent bonds long fibrillar structures can also contribute significantly to gel hardness.

Acknowledgements

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Chapter 7

Control of texture of cold-set gels through programmed bacterial acidification

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Control of texture of cold-set gels through programmed bacterial acidification

Abstract

Cold gelation is a two-step process in which gelation of soluble protein aggregates is induced at ambient temperature by the addition of salt or by lowering the pH. The mechanisms leading to the formation of a protein network are both time- and pH-dependent. It is therefore expected that the final gel properties can be controlled by the time of acidification and final pH reached after acidification. In chemical acidification, these processes cannot be controlled separately (at one defined temperature). Therefore, we applied acid-producing bacteria to independently control the time of acidification and final pH. Gel hardness of bacterially acidified cold-set protein gels of ovalbumin and whey protein isolate could be adjusted by the final pH and by the time of acidification. The pH dependency of the gel hardness is probably related to the net charge of the protein aggregates. Depending on the type of protein, the time-dependent formation of disulphide bonds contributes to the textural properties of cold-set protein gels.

Introduction

Most industrial processing of (globular) food proteins, such as whey proteins and ovalbumin, involves (partial) denaturation of the proteins. Denaturation and the subsequent aggregation of globular food proteins leads to the desired textural food properties. A relatively new process that takes advantage of these changes in the functional properties of the proteins after denaturation is cold gelation. This is a two-step process in which first soluble protein aggregates are prepared by a heat treatment at a pH well above the iso-electric point of the protein and in the absence of salt. After cooling, gelation is induced by lowering the pH or adding salt at ambient temperature (see review of Bryant & McClements, 1989). Cold-set gelation can find application in a wide variety of food products with a delicate flavour and texture (Barbut, 1995). In addition, it can be used as a thickening and gelling agent in a dried form (Thomson, 1994; Resch & Daubert, 2002), to increase efficiency of iron uptake (Remondetto, Paquin & Subirade, 2002) or as a method for encapsulation (Beaulieu, Savaoie, Paquin & Subirade, 2002).

In contrast to heat-induced gelation, the process of acid-induced cold gelation allows control of aggregate properties before inducing gelation. This enabled us to study how aggregate properties determined the final properties of cold-set whey protein isolate (WPI) gels induced by acidification with glucono-δ-lactone (GDL). With this approach, we demonstrated that reduction of the electrostatic repulsion of the WPI aggregates (net electric charge) induced gelation (Alting, De Jongh, Visschers & Simons, 2002). The formation of the initial protein network is primarily determined by physical interactions between the aggregates. The proximity of the aggregates enabled the subsequent formation of covalent disulphide bonds, even at acidic pH values. These disulphide bonds acted as cross-links and stabilised the network, yielding a stronger gel (Alting, Hamer, De Kruif & Visschers, 2000). The formation of disulphide bonds was shown to depend strongly on pH. Both mechanisms are relevant for the application of proteins as a structuring ingredient in food systems.

As these mechanisms are both time- and pH-dependent, it is expected that they can be controlled by the time of acidification and depend on the final pH reached after acidification. In chemical acidification, the time of acidification and final pH cannot be controlled separately (at one defined temperature). The limitations of this use of GDL were earlier recognised for acid-induced milk gels (Heertje, Visser & Smits, 1985; Amice-Quemeneur, Haluk, Hardy & Kratchenko, 1995; Bouzar, Cerning & Desmazeaud, 1997). Therefore, we applied acid-producing bacteria to control the acidification process by varying inoculum size and glucose concentration (carbon source). In

addition, the use of bacterial instead of chemical (GDL) acidification in the process of cold gelation is of practical relevance, because bacterial acidification is often preferred to chemical acidification.

Two types of protein preparations were applied to investigate the effect of specific protein characteristics on the mechanical properties of bacterially acidified cold-set protein gels. We used a bovine-milk-derived protein preparation, WPI and a chicken-egg-derived protein preparation, ovalbumin. The control of the texture of cold-set gels through programmed bacterial acidification, using lactic acid bacteria, of dispersions of aggregates of both types of protein is presented here. *Lactobacillus plantarum* WCFS-1 was used as acid producer for its ability to utilise a large variety of sugars and its possible application in many different fermentations.

Material and methods

Materials. WPI, BiPro, was obtained from Davisco International Inc. (La Sueur, MN). WPI consisted (based on dry weight) of ß-lactoglobulin (74%), α-lactalbumin (12.5%), bovine serum albumin (5.5%) and immunoglobulins (5.5%). The total amount of proteins in the powder is 97.5% and it further contains lactose (0.5%) and ash (2%) (Tuinier, Dhont & De Kruif, 2000). The ovalbumin preparation was obtained from Sigma (albumin chicken egg, A-5503). The protein content was determined by the Kjeldahl method, using a Kjeldahl factor of 6.12. The protein concentration was 88% (Weijers, Visschers & Nicolai, 2002).

Preparation of a dispersion of protein aggregates. Whey protein aggregates were prepared from a solution of 9% (w/w) WPI by heating for 2 h at 68.5ºC following standard procedures (Alting et al., 2000). The amount of native proteins after the heat treatment was determined with a standard assay involving acid precipitation and gel-permeation chromatography (Hoffmann, Roefs, Verheul, Van Mil & De Kruif, 1996). The heat treatment converted more than 95% of the native protein into reactive aggregates and the resulting aggregates had a mean hydrodynamic diameter of 60-70 nm as determined from dynamic light scattering (Alting et al., 2000).

For ovalbumin, a prolonged heating (19.5 h) of a 6% (w/w) solution at 78ºC was used to convert more than 95 % of the native protein into reactive aggregates, determined as described above. The ovalbumin aggregates prepared in this way have a smaller hydrodynamic diameter (approximately 40 nm) compared to the whey protein aggregates as determined from dynamic light scattering (Weijers et al., 2002), assuming that the particles have a spherical shape. For the purpose of this paper differences in shape were not considered.

Both types of protein aggregates prepared in this way contain reactive thiol groups exposed on their surface during the heat treatment. The numbers of thiol groups detected by the use of Ellman's reagent (1959, Alting et al., 2000) are comparable, 0.3-0.4 mM in a 2% (w/w) heated protein solutions of ovalbumin and WPI. These thiol groups are not detectable in solutions of unheated protein (Koseki, Kitabatake & Doi, 1989; Roefs & De Kruif, 1994; Alting et al., 2000).

Organism and growth. A lactic-acid-producing bacterium, *Lactobacillus plantarum* WCFS-1 (Kleerebezem, Boekhorst, Van Kranenburg, Molenaar, Kuipers, Tarchini, et al., 2003) was grown in M17 broth (Merck AG, Darmstadt, Germany) with glucose as carbon source. Late-logarithmic phase cells were harvested by centrifugation. The remaining supernatant was decanted and discarded. The bacteria (pellet) were washed twice with ice-cold double-distilled water and resuspended in double-distilled water to a final volume between 0.5 and 4 % of the volume of the original culture. The dispersions of protein aggregates were inoculated with bacteria to 0.1 to 50% of the total count present in overnight cultures. M17 broth was used to dilute the dispersion to a protein concentration of 2% (w/w). Fermentation was carried out in closed vials at room temperature. During this process the pH was continuously measured.

Gel hardness. Gel hardness was determined by a texture analyser (type TA-XT2, Stable Micro Systems Ltd., Godalming, England). Dispersions of protein aggregates (2%, w/w) were acidified in a beaker in portions of 75 g by the use of *Lactobacillus plantarum* WCFS-1.

Approximately 24 h after the start of the acidification, the acid-induced gels (75 g, 2% (w/w) protein) were penetrated with a stainless steel conical cylinder, with a diameter of 13 mm. A force-distance curve was obtained at a cross-head speed of 0.3 mm/min for a 15 mm displacement, and gel hardness was expressed as the force (g) needed to penetrate 5 mm or 2.5 mm into the gel for cold-set gels of WPI or ovalbumin, respectively. All experiments were performed at least in duplicate and the experimental error was lower than 5%.

Agarose gel electrophoresis. SDS-agarose continuous gel electrophoresis (0.4 % agarose) was performed as described before (Alting et al., 2000) to determine the differences in molecular mass after acid-induced gelation of the dispersion of protein aggregates. Aggregates (in solution or gel) were mixed with 3 parts of 20 mM Bis-Tris buffer (pH 7.0) with 5% SDS and were held at ambient temperature while constantly stirred. After overnight incubation no gel particles could be observed with a standard microscope (at 400 x magnification).

Dynamic light scattering experiments. Dynamic light scattering experiments were performed as outlined by Verheul, Roefs and De Kruif (1998) 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^oC). The apparent diameters of the protein aggregates in solution were calculated from a cumulant fit of the intensity autocorrelation function. Before analysis, samples (0.2% (w/w) protein) were filtered through a low-protein-binding membrane (5 μm; Millex-SV, Millipore Corporation, Bedford, MA., USA). All measurements were performed in duplicate.

Results and discussion

Cold gelation induced by bacterial acidification. Prior to gelation, both dispersions of aggregates were diluted to a final protein concentration of 2% (w/w). At this concentration firm gels can be made from reactive whey protein aggregates by means of chemical acidification using GDL (Alting et al., 2000). The main function of the starter culture applied, Lactobacillus plantarum WCFS-1, is to produce lactic acid through the fermentation of glucose, causing the pH to be gradually reduced. Acid-induced cold-set gels were prepared by adding different amounts of lactic acid bacteria at a constant concentration of glucose or by adding a constant inoculum of bacteria to solutions containing different amounts of glucose. During the acidification process the pH of the gel was continuously measured and 24 h after the start of the acidification, the mechanical gel-hardness was determined.

Effect of final pH. First we examined the effect of the final pH on the gel hardness of cold-set gels prepared by bacterial acidification. **Figure 1A** shows the kinetics of the bacterial acidification of dispersions of whey protein aggregates to which different amounts of glucose had been added (at a constant inoculum of Lactobacillus plantarum WCFS-1). It is important to note that, since the concentration of bacteria added is constant, the initial rate of acidification is the same for all glucose concentrations and that the pH levels off quickly when all the glucose has been used. The final pH reached in the gels is directly determined by the amount of glucose available for fermentation. At pH values lower than 5.9 (corresponding to glucose concentrations higher than 2 mM) a gel is formed during acidification. The same kinetics of acidification were found for the dispersion of ovalbumin aggregates (results not shown).
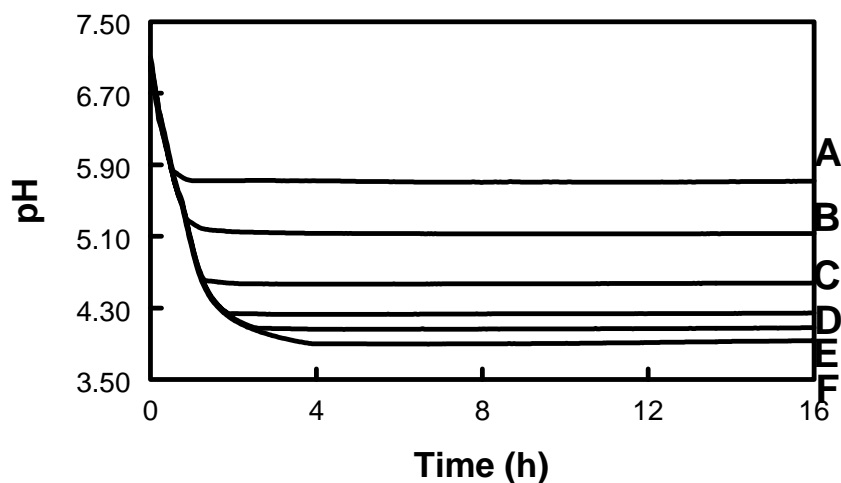
Figure 1A: Glucose-dependent acidification of 2% preheated WPI solutions. The inoculum with *Lactobacillus plantarum* WCFS-1 was 50% of the total count present in overnight cultures. Glucose concentrations were, respectively, 13.3 (A), 22 (B), 33.3 (C), 44.7 (D), 53.3 (E) and 66.7 mM (F).
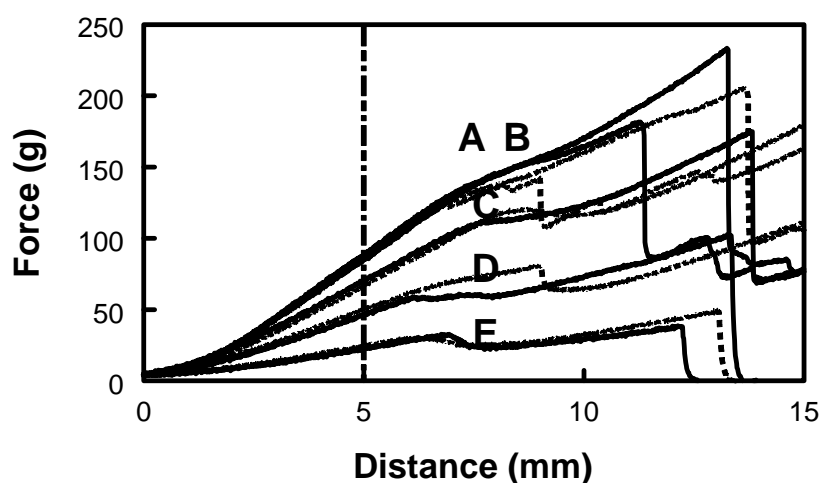
Figure 1B: Large deformation properties of bacterially acidified cold-set WPI gels (2% protein) with different final pH values (see Fig 1A). The final pH values were, respectively, 5.14 (A), 4.58 (B), 4.25 (C), 4.09 (D) and 3.94 (E). At pH 5.72 no gel was formed. The dashed lines represents the results of duplicate experiments.

The mechanical strength of the gels formed during the fermentation depended directly on their final pH. **Figure 1B** shows typical force-distance curves obtained with the texture analyser. Texture analysis showed a typical behaviour of the cold-set gels as was previously found for chemically acidified cold-set gels. Some strain hardening occurs during the initial compression. To further analyse the results we plotted the force at a pre-set penetration (**Figure 1C**). With decreasing final pH, gel hardness decreases. A plateau seems to exist between pH 4.5 and 5.2 for the WPI gels. Below a pH of 4.5 the gel strength starts to decrease rapidly.

Gels formed by bacterial acidification of 2% (w/w) dispersions of ovalbumin solutions were found to be much weaker than 2% (w/w) WPI gels formed under the same conditions (**Figure 1C**). Note that the penetration distance was different. A plateau in the gel hardness as observed for the WPI gels was also observed for the ovalbumin gels extending from pH 4.2 to 5.2. The decline at lower pH values observed for the cold-set WPI gels was less pronounced for the cold-set ovalbumin gels.
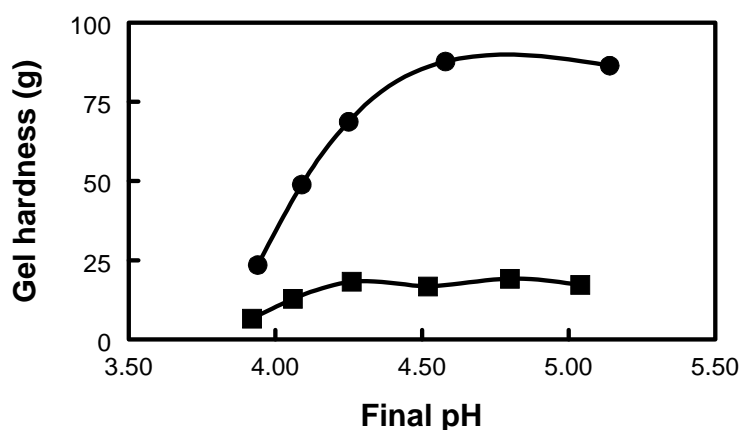

Figure 1C: Gel hardness expressed as the force needed to penetrate a distance of 5 mm (WPI)(see Figure 1B) or 2.5 mm (ovalbumin) as a function of the final pH for a 2% (w/w) dispersion of WPI-aggregates (filled circles) and ovalbumin-aggregates (filled squares).

As the acid-induced formation of a protein network is driven by the reduction of electrostatic interactions around the iso-electric point (Alting et al., 2002), the gel hardness is expected to have its maximum strength around the pH value of 5.1 and 4.7 for WPI and ovalbumin, respectively. A decrease or increase in the final pH away from the iso-electric point will increase the charge on the protein aggregates, resulting in more repulsion between the building particles of the gel network, and therefore in gels with altered properties. The pH-dependence of the gel hardness of cold-set WPI gels was earlier determined by Ju and Kilara (1998). In contrast to the bacterial acidification applied in this study, they varied the resultant pH by adding different amounts of GDL (0.2 to 2.0%), resulting in a final pH ranging from 3.5 to 5.7. As the protein concentration was much higher in their experiments, the gels were much stronger, but the pH-dependence of the gel hardness was in agreement with our results on bacterial acidification. As the time of acidification in their experiments probably varied with the amount of GDL added, it can be concluded that the effect of the final pH on the gel hardness dominated an eventual effect of the time of acidification on the gel hardness.

It is known that during acid-induced cold gelation of WPI aggregates chemical cross-links (disulphide bonds) are formed between the aggregates (Alting et al., 2000). The increase in aggregate size, caused by the formation of disulphide cross-links between the initial aggregates, was demonstrated by a decrease in migration velocity during SDS-agarose electrophoretic analysis. This electrophoresis technique was shown to be suitable to separate protein (aggregates) with hydrodynamic diameters ranging from 3 (monomeric protein) to approximately 250 nm. The formation of disulphide bonds in cold-set WPI gels corresponds to an increase in gel hardness. To determine if disulphide bonds were also formed during acid-induced cold gelation of a dispersion of ovalbumin aggregates, gels were resolubilized in a buffer containing SDS and analysed using SDS-agarose electrophoresis. No decrease in migration velocity of ovalbumin aggregates was observed, as was previously found for dispersions of WPI aggregates after acid-induced gelation (Alting et al., 2000). Absence of disulphide bond formation may explain the difference in pH-dependence of the gel hardness between cold-set gels of WPI and ovalbumin (**Figure 1C**). Probably the initial difference in aggregate size before gelation is induced between the WPI and the ovalbumin aggregates cannot explain the difference in gel hardness. Elsewhere, we have shown that the effect of disulphide bond formation dominated over the effect of different structural properties (Alting, Hamer, De Kruif, Paques & Visschers, 2003a). Despite their inability to form extensive disulphide bonds during acid-induced gelation, acidification of the ovalbumin aggregates resulted in gels from which the strength could be measured.
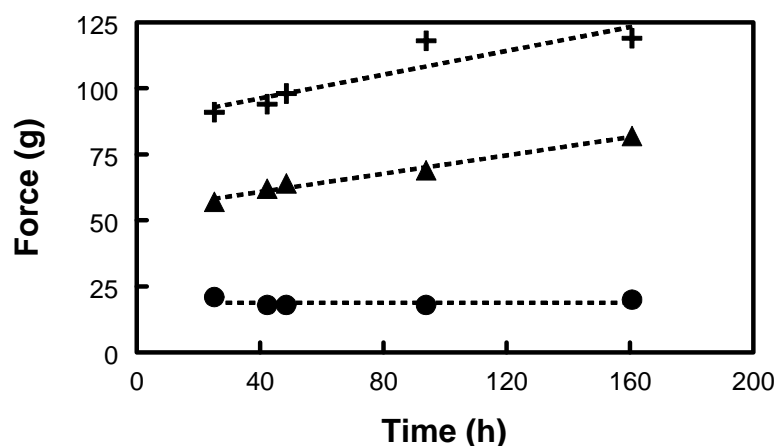

Figure 2: Development of the gel hardness with time of bacterially acidified cold-set WPI-gels with different final pH values, final pH 3.9 (66.7 mM glucose, circles), final pH 4.2 (44.7 mM glucose, triangles), final pH 5.0 (22 mM glucose, crosses). The inoculum with *Lactobacillus plantarum* WCFS-1 was 50% of the total count present in overnight cultures.

Figure 2 shows the development of the gel hardness with time of cold-set WPI gels with different final pH values. The hardness of cold-set WPI gels with a final pH of 3.9 remained constant with time. However, the hardness of gels with higher final pH values, respectively 4.2 and 5, increased with time. This increase in gel hardness with time was earlier observed for chemically acidified cold-set WPI gels (Alting et al., 2000) and chemically acidified gels of heated milk (Vasbinder, Alting, Visschers, & De Kruif, 2003). In both studies the increase was attributed to the

additional formation of disulphide bonds with time. The use of succinylated WPI aggregates (lowered iso-electric point) enabled the preparation of cold-set gels at much lower pH-regions. These experiments demonstrated that the formation of disulphide bonds was pH-limited and indeed did not occur at pH values lower than 3.5-4 (Alting et al., 2002). The microstructure formed after acid-induced gelation will not be further stabilised by chemical cross-links, and only physical interactions will contribute to the mechanical properties, leading to relatively weaker gels and possible syneresis.

Effect of inoculum-dependent acidification kinetics. The presence of a pH region in which the gel hardness does not depend on the final pH of the gel allowed us to determine the effect of different acidification profiles on the final strength of these gels. In general, the final pH values of the gels were within this linear region, 4.8 ± 0.1. Changing the initial amount of bacteria that was added to the aggregate solutions varied the time of acidification. In contrast to the previous experiments, the glucose concentration was kept constant. The amount of glucose was chosen such that the final pH that was reached during fermentation was limited by the amount of glucose. The time of acidification was lower when the size of the inoculum was smaller (**Figure 3A**). The time taken to reach a pH of 5.5 varied from 220 to 750 min for an inoculum of 10% and 0.5% respectively.
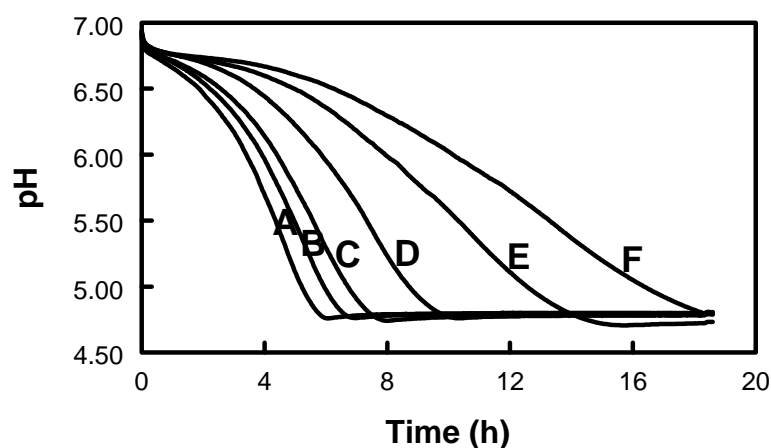
Figure 3A: Inoculum dependent acidification of 2% preheated WPI solutions. The concentration of glucose was 26.7 mM. The inoculum sizes with *Lactobacillus plantarum* WCFS-1 were 10 (A), 7.5 (B), 5.0 (C), 2.5 (D), 1.0 (E) and 0.5 (F) of the total count present in overnight cultures, respectively.

After the gels were set and a stable pH was reached (after 24 h) the mechanical strength of the gels was measured (**Figure 3B**). In **Figure 4** the relation between the time of acidification and final gel hardness is given for WPI and ovalbumin gels. The final gel hardness depended on the time of acidification for both WPI and ovalbumin gels. At short acidification times (short t½) relatively strong gels were formed. Note that at the time the hardness was determined these gels had been longer in the gel state.
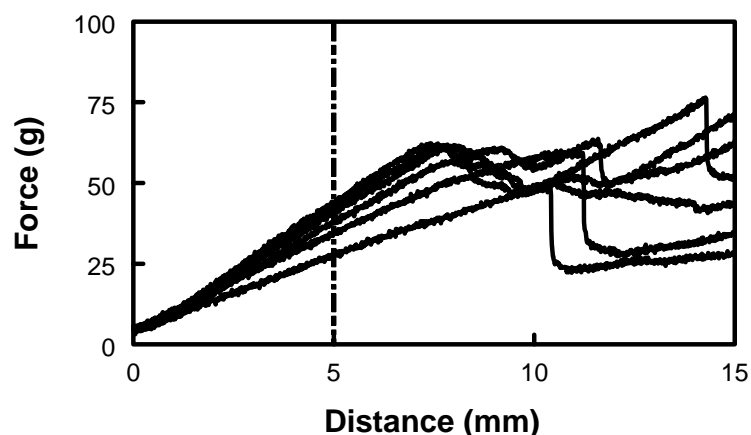
Figure 3B: Large deformation properties of bacterially acidified cold-set WPI gels (2% protein) with different inoculum sizes (see Fig. 3A) (final pH values 4.7-4.8). The inoculum sizes with *Lactobacillus plantarum* WCFS-1 were 0.5 (lower curve), 1.0, 2.5, 5.0, 7.5 and 10% (upper curve) of the total count present in overnight cultures, respectively.

From chemical acidification studies at ambient temperature we know that the additional formation of disulphide bonds takes place on a time scale of hours (Alting et al., 2000). Gels prepared with a small inoculum size (0.5%) dissolved more easily in an SDS-containing electrophoresis buffer and resulted in less turbid solutions, whereas gels prepared with a larger inoculum size (10%) dissolved more slowly and resulted in more turbid solutions. SDS-agarose electrophoresis could not reveal differences, since the protein structures present in both samples were not able to enter the agarose gel matrix. Dynamic light scattering indeed confirmed that the hydrodynamic diameters of the protein structures present were larger than 250 nm, the upper limit for the separation of protein structures using 0.4% agarose electrophoresis under the applied conditions. The mean hydrodynamic diameters determined by dynamic light scattering in cold-set gels prepared with an inoculum size of 0.5% and 10% were 256 ($\pm$ 3) nm and 929 ($\pm$ 39) nm, respectively. In addition, the contribution of the time-dependent formation of disulphide bonds to the gel hardness of bacterially acidified cold-set WPI was already clearly shown (**Figure 2**) and was previously demonstrated for GDL-acidified milk gels (Vasbinder et al., 2003). These results strongly suggest that disulphide cross-linking of protein aggregates is time-dependent (time-scale of hours), with the initial hydrodynamic diameter of 60-70 nm growing into larger protein structures, that clearly contribute to the hardness of cold-set WPI gels. A similar mechanism could also explain the observation in caprine milk of an inoculum-size-dependent effect on the gel hardness (Vlahopoulou, Bell & Wilbey, 1994).

For ovalbumin no formation of disulphide bonds was observed during the gel state, which probably explains why there was little or no dependence on the final pH. However, we do observe a dependence of the final gel hardness on the time of acidification (**Figure 4**). We speculate that when the ovalbumin system is longer in the gel state more structural rearrangements occur, leading to a more efficient packing, more bonds and therefore stronger gels when acidification proceeds rapidly. Indeed syneresis, which is an indicator for the occurrence of structural rearrangements, was more pronounced for the ovalbumin gels than the WPI-gels. For the latter we have hypothesised

that the additional formation of disulphide bonds reduced or prevented the occurrence of rearrangements and syneresis in GDL-acidified cold-set WPI gels (Alting, Hamer, De Kruif & Visschers, 2003b).
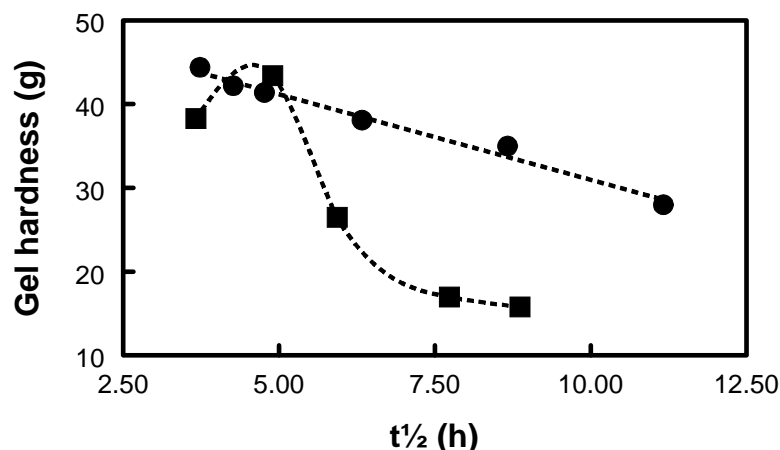
Figure 4: Gel hardness as a function of the time of acidification for cold-set WPI gels (circles) and for cold-set ovalbumin gels (squares). t½ was obtained from the acidification curves of Fig. 3A.

In conclusion, as far as we know this is the first time that (lactic-acid-producing) bacteria have been successfully applied as acidifiers to induce the cold gelation of WPI and ovalbumin. Gel hardness of bacterially acidified cold-set protein gels could be controlled by the final pH or by the time of acidification. The pH dependency of the gel hardness is most likely related to the net charge of the protein aggregates. Depending on the type of protein, the time-dependent formation of disulphide bonds contributes to the final mechanical properties of the cold-set protein gels. In the light of shortening production times of acidified food products these results could be of relevance for the food industry. Since the disulphide cross-linking takes place at ambient temperature and at a time-scale of hours it is highly relevant for textural properties of cysteine-containing acidified food products, such as yoghurt (Hashizume & Sato, 1988; Britten & Giroux, 2001; Vasbinder et al., 2003).

Acknowledgements

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Texture of acid milk gels: formation of disulfide cross-links during acidification

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Texture of acid milk gels: formation of disulfide cross-links during acidification

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Abstract

Denaturation of whey proteins during pasteurization of milk results in the formation of whey protein aggregates and whey protein-coated casein micelles. After cooling a substantial number of thiol groups remains exposed. Formation of larger disulfide-linked protein structures during acidification at ambient temperature was demonstrated by analytical methods. The time-dependent formation of these structures attributed significantly to the mechanical properties of acid milk gels, resulting in gels with an increased storage modulus and hardness. Addition of the thiol-blocking agent *N*-ethylmaleimide prevented the formation of disulfide-linked structures. The mechanical properties are shown to be the result of the contribution of denatured whey proteins to the protein network as such and the additional formation of disulfide bonds. Surprisingly, these sulfhydryl group-disulfide bond interchange reactions take place at ambient temperature and under acidic conditions. Therefore, the disulfide cross-linking is highly relevant for textural properties of acid-milk products, like yoghurt.
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Keywords: Disulfide bonds; Thiol groups; Whey proteins; Heated milk; Acidification; Texture

1. Introduction

Historically, acidification of heated milk was applied as a preservation technique. Nowadays, acidified products like yoghurt are highly appreciated for their texture, taste and health properties. During yoghurt preparation, the milk is subjected to a heat treatment before acidification. Therefore, production of yoghurt can be seen as a two-step process.

1.1. Heat treatment of milk (step 1)

On heat treatment at temperatures higher than 70°C the major whey proteins, i.e. β-lactoglobulin (β-lg) and α-lactalbumin (α-lac), denature (Dannenberg & Kessler, 1998). The temperature-induced conformational change of β-lg results in the exposure of both hydrophobic parts

of the polypeptide and reactive thiol groups. These reactive thiol groups can form disulfide links with other reactive thiol groups or disulfide bridges as present in α-lac, β-lg, BSA, κ- and αs_2-casein through thiol group/disulfide bond interchange reactions. During the heating of milk, mainly β-lg covalently interacts with κ-casein present at the exterior of the casein micelles (Corredig & Dalgleish, 1996; Jang & Swaisgood, 1990; Singh, 1993). However, significant quantities of α-lactalbumin and the minor whey proteins also can interact with the casein micelles (Corredig & Dalgleish, 1996; Oldfield, Singh, Taylor, & Pearce, 2000). Additionally, soluble disulfide-linked whey protein aggregates are formed (Oldfield et al., 2000; Anema & Klostermeyer, 1997). So, heated milk is a complex mixture of native and denatured whey proteins and casein micelles in which the denatured whey proteins occur either as whey protein aggregates or as whey protein (aggregates) associated with the casein micelles. A significant amount of thiol groups will remain active after cooling the milk to ambient temperature (Hashizume & Sato, 1988; Kirchmeier,

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El-Shobery, & Kamal, 1984; Kirchmeier, Kamal, & Klostermeyer, 1985; Hong, Guthy, & Klostermeyer, 1984; Guincamp, Humbert, & Linden, 1993).

1.2. Acidification of heated milk (step 2)

1.2.1. Effect of the presence of whey proteins

Acidification of milk towards the iso-electric point of the caseins, i.e. pH 4.6, lowers the stability of the κ-casein brush on the surface of the casein micelles (Tuinier & De Kruif, 2002). The casein micelles loose their steric stabilization and Van der Waals attraction causes flocculation. Heat treatment of milk prior to acidification of milk at temperatures ranging from 20°C to 40°C changes the gelation properties markedly compared to those of unheated milk. Heat treatment has caused a shift in gelation pH towards higher pH values (Heertje, Visser, & Smits, 1985; Horne & Davidson, 1993; Lucey, Tet Teo, Munro, & Singh, 1997; Vasbinder, van Mil, Bot, & De Kruif, 2001). The final gel formed has an increased gel hardness, higher storage modulus (G') (Lucey et al., 1997; Lucey, Tamehana, Singh, & Munro, 1998; Parnell-Clunies, Kakuda, deMan, & Cazzola, 1988; van Vliet & Keetels, 1995) and shows less susceptibility to syneresis (Dannenberg & Kessler, 1988a, b). These effects are related to whey protein denaturation and the whey protein coating of the casein micelles, causing an increase of the pI from 4.6 (pI casein micelles) to 5.2 (pI whey proteins) (Vasbinder et al., 2001). Electron microscopy revealed that heat treatment of milk changed casein micelles into micelles with appendages composed of whey proteins on the surface. These whey protein coated micelles are thought to cause the increased gel hardness and decreased syneresis as they prevent coalescence of the micelles and increase the number of contact points between the micelles (Davies, Shankar, Brooker, & Hobbs, 1978; Heertje et al., 1985; Mottar & Bassier, 1989).

1.2.2. Effect of thiol group/disulfide bond interchange reactions

It seems likely that the exposed thiol groups formed during heat treatment, and still detectable after cooling, have an additional effect on gel strength. Lucey et al. (1998) demonstrated that addition of a thiol-blocking agent, N-ethylmaleimide (NEM), after heat treatment, but prior to acidification at 30°C, caused a decrease of 15% in the final storage modulus (G'). It was not further investigated whether this was due to inhibition of thiol group/disulfide bond interchange reactions or non-specific interference of NEM with gel formation (Lucey et al., 1998). As far as we know this is the only experiment performed under conditions close to ambient temperature. Hashizume and Sato (1988) and Goddard (1996) performed more extensive studies on

the relation between thiol groups and gel strength, but the acid coagulation took place at 60°C (Goddard, 1996) or at temperatures between 60°C and 80°C (Hashizume & Sato, 1988). At these temperatures the processes of whey protein denaturation and acid-induced gelation are intertwined. Additionally, these temperatures are much too high for lactic acid bacteria to fulfill their essential role in the production of acidified milk products, i.e. production of lactic acid and extracellular polysaccharides. In daily practice, production of yoghurt takes place at far lower temperatures (20–40°C); here it is a two-step process where denaturation and acid-induced gelation are clearly separated. Therefore, it is questionable whether the disulfide exchange reactions observed by both Hashizume and Sato (1988) and Goddard (1996) will still take place at an acidification temperature of 20°C and at a time scale relevant for yoghurt production, i.e. 24–48 h. In this article, we demonstrate the time-dependent development of disulfide bridges during acid-induced gelation (20°C) of heated milk, as well as their effect on the mechanical properties of acid milk gels. This work will contribute to a better understanding of thiol group/disulfide bond interchange reactions during acid-induced gel formation under temperature conditions relevant for the production of yoghurt and demonstrates their importance for texture formation.

2. Material and methods

2.1. Reagents and chemicals

Glucono-δ-lactone (GDL), 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), sodium dodecyl sulphate (SDS), dithiothreitol (DTT), and NEM were obtained from Sigma Chemicals (St. Louis, MO, USA). Electrophoresis-grade agarose was obtained from Life Technologies (Paisley, Scotland). Phastgel blue R tablets were from Pharmacia Biotech (Uppsala, Sweden). Fresh skim milk was obtained from the NIZO pilot plant (Ede, the Netherlands). Transglutaminase (Ca^{2+}-independent) was supplied by Ajinomoto Co. Inc. (Japan).

2.2. Preparation of reconstituted skimmed milk

Reconstituted skim milk was prepared by dissolving 10.45 g low-heat milk powder (Nilac; NIZO food research, Ede, the Netherlands) in 100 g distilled water while gently stirring (final protein concentration, 3.5% w/w). The milk was stirred at 45°C for 1 h. To prevent bacterial growth 0.02% sodium azide was added and the milk was kept overnight at 4°C before use. The initial pH of the milk was 6.67 (± 0.01).

2.3. Preparation of whey protein-free reconstituted skim milk

Whey protein-free reconstituted skim milk (0.02% (w/w) native whey protein) was prepared by dissolving 8.78 g whey protein-free milk powder (prepared by ultra- and micro-filtration) in 91.22 g distilled water. Further treatment was identical to preparation of skimmed milk as described above.

2.4. Intra-micellar cross-linking of the micelles by transglutaminase

The required amount of reconstituted skim milk (stored at 4°C) was stirred for 2 h at 20°C and then incubated for 1 h at 40°C. A 2% transglutaminase solution (activity 20 U g^{-1}) was used to reach a final activity in the milk of 50 U g^{-1} protein (protein content of milk is 3.5%). This solution was mixed and incubated for 1 h at 40°C. The solution was transferred to glass tubes (5 mL per tube) and heat treated for 25 min at 90°C. After cooling under tap water to 20°C the milk was either used directly or stored overnight at 4°C.

2.5. Sample preparation: heat treatment

The required amount of fresh and reconstituted skim milk and whey protein-free reconstituted skim milk (stored at 4°C) was stirred for 2 h at 20°C, followed by a heat treatment of 10 min at 90°C in aliquots of 5 mL. After heating the samples were cooled under tap water.

2.6. Blocking and determination of reactive thiol groups

The number of accessible thiol groups in milk was determined essentially according to Hashizume and Sato (1988), using DTNB, also known as Ellman's reagent (Ellman, 1959). Shortly, to 5 mL milk, 4 mL of 50 mM imidazol-buffer pH 7 was added and 1 mL of a 1 mg ml^{-1} solution of DTNB in the same buffer. After 5 min, 4 g of ammonium sulfate was added to coagulate the milk proteins that were subsequently separated by centrifugation. From the extinction at 412 nm, the number of thiol groups was calculated using a molar extinction coefficient for 2-nitro-5-mercapto-benzoic acid of 13,600 M^{-1} cm^{-1}. The assay was performed in the absence of detergents like urea or SDS, since under these conditions only the thiol groups of interest, those at the surface of the (coated) micelles, were determined. To block the thiol groups in heated milk, NEM was added in a final concentration of 5 mM. The effectiveness of this treatment was confirmed as described above.

2.7. Sample preparation: acidification by GDL in absence or presence of NEM

The different milks were kept at 20°C for 75 min. NEM was added in a final concentration of 5 mM prior to acidification with 1.2% GDL. After addition of GDL the milk was stirred gently for 2 min and incubated at 20°C. A pH of around 4.6 is reached after approximately 24 h. Diffusing wave spectroscopy was applied to determine the gelation point as described by Vasbinder et al. (2001).

2.8. Solubilization of acid-induced gels

Solubilization of the acid-induced gels was done according to Alting, Hamer, De Kruif, and Visschers (2000). After 24 h of incubation with GDL, the gel formed was mixed with 3 parts (w/w) of 20 mM bis-tris buffer and 5% SDS (pH 7.0). The samples were held overnight at ambient temperature, while being constantly stirred. In some experiments, a treatment with the disulfide-reducing agent DTT (0.05%) was carried out to reduce all the disulfide bonds in solubilized samples.

2.9. Agarose gel electrophoresis

SDS-agarose gels were prepared with 0.7% agarose for milk samples and 0.4% agarose for cross-linked systems. The agarose gel electrophoresis was carried out according to Alting et al. (2000). Briefly, the electrophoresis buffer consisted 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. The milk samples and resolubilized gel samples were mixed with 1 part of 20 mM bis-tris buffer, 5% SDS (pH 7.0). The samples were held overnight at ambient temperature. 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 50 V for approximately 2 h and stained with Phastgel blue R.

2.10. Gel hardness

Large deformation tests were performed with a texture analyser (type TA-XT2, Stable Micro Systems ltd., Godalming, England) (empirical "texture" test). The acidification took place in a beaker containing 100 g milk. After 24 h of incubation, the acid-induced gels were penetrated with a wire mesh-device. The mesh consisted of four blades ($45 \times 1.5 \times 2$ mm^3) of stainless steel arranged in a double cross. A force–time curve was obtained at a crosshead speed of 0.3 mm s^{-1} 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). All experiments were at least performed in duplicate and the experimental error was lower than 10%.

2.11. Storage modulus G'

The storage modulus G' was determined with a Carrimed Rheometer (type CSL^2 500, TA instruments N.V./S.A.-Benelux). Measurements were performed using a conical concentric cylinder measuring unit (inner radius 8.60 mm, outer radius 9.33 mm). Directly after the addition of GDL, samples were brought into the measuring unit and covered with a thin layer of paraffin oil to prevent evaporation. The acidification took place in the rheometer at 20°C and G' was followed with time. Measurements were carried out every 12 min at 1 rad s^{-1} and at a constant strain of 1%, which is in the linear region. The small amplitude oscillatory measurements were at least performed in duplicate with an experimental error lower than 10%.

2.12. Confocal scanning laser microscopy

Imaging was performed using a Leica confocal scanning laser microscope, type TCS-SP, configured with an inverted microscope, and an Ar–Kr laser for single-photon excitation (Leica microsystems, Rijswijk, The Netherlands). 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.

2.13. Dynamic light scattering experiments

Dynamic light scattering experiments were performed as outlined by Verheul, Roefs, and De Kruif (1998) 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 thermostatted by a Joule–Peltier thermostat (20°C). The apparent diameter of the micelles 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, USA). All measurements were performed in duplicate.

3. Results and discussion

3.1. Formation of reactive thiol groups during heat treatment of milk

A heat treatment of 10 min at 90°C caused more than 90% denaturation of the whey proteins (Vasbinder et al., 2001) and resulted in a significant exposure of reactive thiol groups in reconstituted and fresh skim milk, respectively, 0.07 and 0.10 mM. In the whey protein-free reconstituted skim milk, thiol groups were not detectable after the same heat treatment. Addition of 5 mM NEM to the different types of milk resulted in a total blocking of the thiol groups, as the absorbance measured at 412 nm was identical to the unheated milk samples.

In agreement with previously reported work (Hashizume & Sato, 1988; Kirchmeier et al., 1984, 1985; Hong et al., 1984; Pofahl & Vakaleris, 1968) heat treatment of milk caused the exposure of reactive thiol groups. The lower number of thiol groups determined in reconstituted skim milk can be due to differences in the degree of whey protein denaturation (Anema & Lloyd, 1999) caused by the additional spray drying process to another heat treatment of the milk before spray drying or to differences in whey protein levels between the milks. As reported previously by Pofahl and Vakaleris (1968), in the absence of whey proteins (whey protein-free milk) only a negligible number of free thiol groups could be detected after 10 min of heat treatment. The results demonstrate the relevance of whey proteins in the formation of reactive thiol groups during heating of milk.

Addition of 5 mM NEM after the heat treatment and prior to acidification was sufficient to block all detectable thiol groups in the different types of heated milk studied. In the literature, different concentrations of NEM in milk are proposed to inactivate the thiol groups formed during heat treatment. Lucey et al. (1998) reported a relatively high concentration of 20 mM. However, a much lower quantity of NEM was reported by Goddard (1996), who demonstrated that a concentration of 0.075 mM NEM was sufficient to exclude the effect of disulfide formation on the gel strength. Alting et al. (2000) observed that 0.5 mM NEM was sufficient to block a concentration of 0.35 mM thiol groups in a solution of whey protein aggregates. The number of detectable thiol groups in the present milk systems is only a fraction of this.

3.2. Effect of heat treatment on the casein micelle system

In order to investigate the effect of heating on the mechanical properties of acid-induced casein gels in the absence of whey proteins, large deformation experiments with acidified whey protein-free reconstituted

skim milk gels were performed. Fig. 1 clearly shows that the force–distance curves for wire mesh penetration for unheated and heated whey protein-free reconstituted acid skim milk gels are identical. In this study, no effect of heat treatment was observed on the large deformation studies performed on acid gels of whey protein-free milk. Diffusing wave spectroscopy experiments had already shown that the start of gelation of whey protein-free milk was not affected by a similar heat treatment (Vasbinder et al., 2001). Therefore, we can conclude that in this study the gel formation and mechanical properties of casein micelle-based gels are not affected by eventually heat-induced changes in the casein micelle system as earlier observed by Schreiber (2001) in rennet-induced gels of milk heated at ultra high temperatures (100–140°C).

3.3. Effects of NEM on mechanical properties of acid-induced unheated milk gels

To investigate a possible non-specific effect of the reactive thiol group blocker NEM on the mechanical properties of acidified milk gels, non-heated reconstituted skim milk with and without NEM was compared. Reactive thiol groups were not present in unheated milk, and therefore specific effects of NEM can be excluded. The mechanical properties of the gels were monitored using both non-destructive, dynamic measurements within the linear region and destructive large deformation measurements. Figs. 2 and 3 clearly show that for the acidified gels of both reconstituted and fresh skim milk without heat treatment, there were no non-specific effects of NEM on mechanical properties, since both the cure curve (development of the storage modulus with time) (Fig. 2) and the force distance–curve (Fig. 3) proceed identically with and without the addition of

Fig. 2. Development of the storage modulus with time for acidified non-heated (squares) and heated (circles) reconstituted skim milk in the absence (open symbols) and presence of NEM (closed symbols).

Fig. 3. Effect of thiol blocking on the large deformation properties of unheated (squares) and heated (circles) reconstituted skim milk in the absence (open symbols) and presence of NEM (closed symbols) after 24 h of acidification.
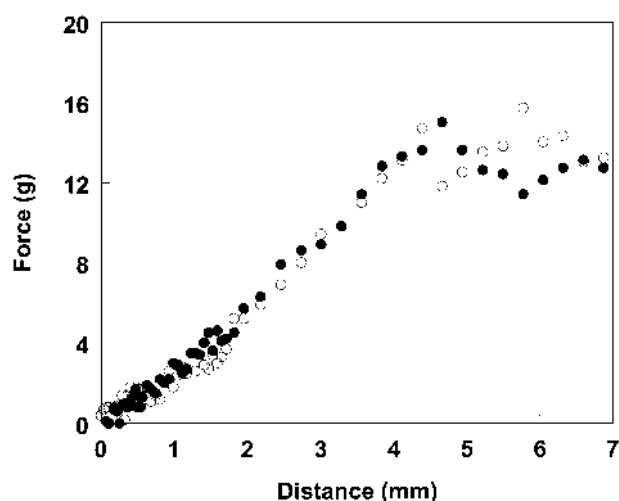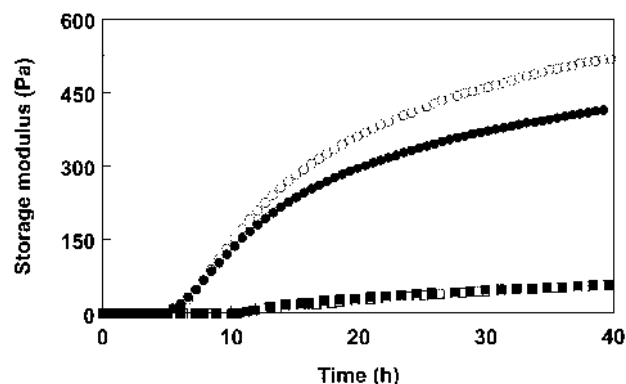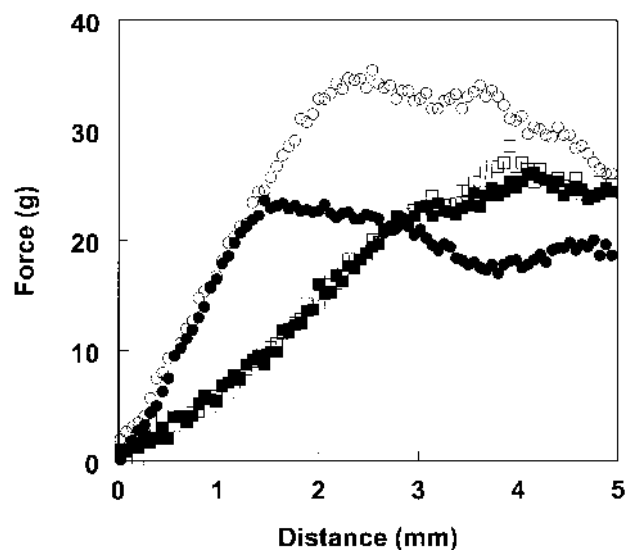

Fig. 1. Effect of heat treatment on the large deformation properties of whey protein-free reconstituted skim milk in the absence (open circles) and presence of NEM (closed circles) after 24 h of acidification.

NEM. In addition, no changes in both the acidification profile and the pH of gelation were observed after the addition of NEM.

3.4. Effect of whey protein denaturation on mechanical properties

As already mentioned, heat treatment of milk results in the denaturation of whey protein in whey protein aggregates and in whey protein (aggregates) associated with the casein micelles. To study the effect of this denaturation on the gel strength of acid milk gels without the interference of the additional formation of disulfide bonds during acidification, free thiol groups present after heat treatment were blocked by the

application of NEM. Although, the pH of gelation has changed after heating (Vasbinder et al., 2001), no differences in gelation pH between the non-treated and the NEM-treated (heated) milk were observed. The mechanical properties of the acidified milk gels were monitored using both non-destructive, dynamic measurements and destructive large deformation measurements. A gelation time of 11 and 5.5 h was observed for unheated milk and heated reconstituted skim milk treated with NEM, respectively, which was followed by a steady increase of the storage modulus, G', with time (Fig. 2). In these measurements, G' represents the elastic shear modulus of a gel. After 40 h of acidification, heated reconstituted skim milk treated with NEM reached an approximately 7 times higher G' value than unheated reconstituted skim milk. Fig. 3 shows the results of a large deformation study on acidified gels of unheated milk (open squares) and heated reconstituted skim milk with NEM (closed circles). Although gel hardness, expressed as the force (g) at the maximum of the force–distance curve (Bourne, 1978), did not differ for NEM-treated heated milk and unheated milk, the slope of the force–distance curve of unheated and heated milk clearly differed. This slope relates to the elasticity of the gel. Qualitatively this agrees with the differences in elastic behaviour observed in the small deformation experiments.

The observed decrease in gelation time of NEM-treated heated milk compared to unheated milk is thought to be due to the whey protein coating of the casein micelles. The coating caused a change in the pI of the casein micelles from the pI of casein (4.6) towards that of whey protein (5.2). This has a clear effect on the gelation pH (Horne & Davidson, 1993; Vasbinder et al., 2001; Heertje et al., 1985; Lucey et al., 1997) and subsequently on the gelation time as the same amount of GDL is applied. The elastic modulus of the gel clearly increases due to whey protein denaturation as the gels reach higher G'-values and have a steeper slope in the force–distance curves. The amount of additional protein available for structuring after heat treatment (approximately 20% of the total protein) is far too small to explain this increase. In electron microscopy pictures, the formation of appendages on the casein micelles during heat treatment could be observed and it is generally believed that these interfere with the aggregation of the casein micelles and increase the interactions between the casein micelles (Davies et al., 1978; Heertje et al., 1985; Mottar & Bassier, 1989), which explains the increase in elasticity of the gels. As the formation of disulfide bridges between the particles during the gel state was prevented by the NEM treatment in both cases only physical interactions (electrostatic, hydrophobic, hydrogen bonds) will occur, which finds expression in the identical gel hardness obtained in the large deformation experiments. In conclusion whey protein

denaturation clearly enhances elastic properties of acid milk gels.

3.5. Effect of disulfide bridge formation on the gel strength during acid-induced gelation

Fig. 2 shows that the cure-curves of the non-treated (open circles) and NEM-treated (closed circles) heated reconstituted skim milk are identical at the start, and show a steady increase with time. After 10 h of acidification, a difference in G' value is observed and increases with time. After 48 h of acidification at ambient temperature the non-treated milk reaches a 20% higher G' value. With large deformation experiments, a 30% increase in gel hardness was observed after 24 h of acidification, while the slope was apparently not affected (Fig. 3). With heated fresh skim milk a significant higher gel hardness was determined (50 g) than with heated reconstituted skim milk (35 g). Both NEM-treated milks had a gel hardness of 23 g (graph not shown). Confocal scanning laser microscopy was applied to the different acid milk gels to observe potential differences in the microstructure. Neither the heat treatment nor the modification of the thiol groups were found to have an effect on the microstructure at a micrometer length scale (results not shown).

A comparison of heated milk with and without NEM treatment revealed the clear contribution of disulfide bonds to both the storage modulus and the gel hardness of acid-induced milk gels. The increase in the elasticity of the gels (20%) is in agreement with the results of Lucey et al. (1997), who found a similar increase. The slope for acidified heated milk was hardly affected by the NEM treatment, suggesting that the storage modulus is a more sensitive parameter to study the elastic behavior. However, the gel hardness seems to be a sensitive parameter to study disulfide bridge formation under acidic conditions. The experiments with fresh skim milk demonstrated that a higher number of thiol groups also resulted in a higher gel hardness. The amount of free thiol groups after heating seems to correlate with the gel hardness.

3.6. Effect of time on gel hardness

Fig. 4 shows the development of the gel hardness with time for heated fresh skim milk at ambient temperature. The NEM-treated milk reaches a plateau value of less than 30 g after approximately 30 h of acidification. The non-treated milk reaches a significantly higher gel hardness value than the NEM-treated milk. After 35 h of acidification, a value of 60 g is reached and the hardness steadily increases during further incubation to 90 g after 75 h. At this point there is still no levelling off.

Since in NEM-treated milk the ability to form disulfide cross-links is absent during acidification, the
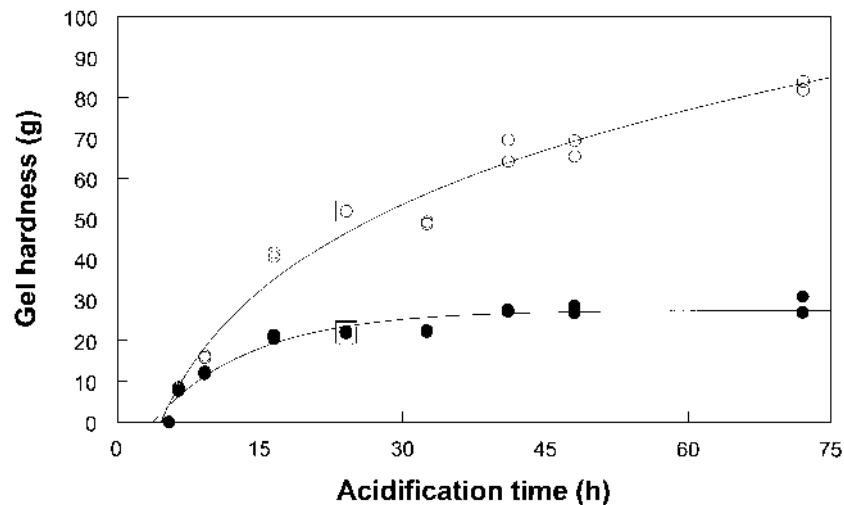
Fig. 4. Development of the gel hardness with time for heated fresh skim milk in the absence (open symbols) and presence (closed symbols) of NEM. The lines are drawn to guide the eye. The squares mark the gel hardness after 24 h of acidification of which the complete force–distance curves are shown in Fig. 3.

gel hardness–time curve represents the formation of a protein network built up of particles connected via physical interactions. The time needed to reach a plateau value seemed to correspond with the time needed to completely hydrolyse GDL, i.e. to reach the final pH of around 4.6. From that point on the environmental conditions will be constant and no further increase of gel hardness was observed. This would indicate that no further structural rearrangements take place which have an influence on the gel hardness. The additional contribution of covalent disulfide bonds between the particles is clearly seen on top of the formation of a physically linked protein network in the absence of NEM. This process is time dependent and proceeds steadily after the final pH is reached. Since it occurs on a time scale of hours it is relevant for the textural properties of yoghurt.

3.7. Formation of disulfide linked structures during gelation

3.7.1. SDS-agarose gel electrophoresis

The SDS-agarose gel electrophoresis was shown to be suitable to separate protein (aggregates) with a diameter ranging from 3 (monomeric protein) to approximately 250 nm. With this technique much larger protein structures can be determined than with classical polyacrylamide gel electrophoresis (PAGE) (Alting et al., 2000). Fig. 5 shows that before gelation no effect is observed of the NEM treatment on the migration velocity of the protein bands (lanes 1 and 2). Since the agarose electrophoresis gels were run without stacking gel, diffuse bands were observed. A clear effect of the addition of NEM is observed after gelation. Only in the absence of NEM are slower migrating structures observed (bracket a in lane 3). In both cases, addition
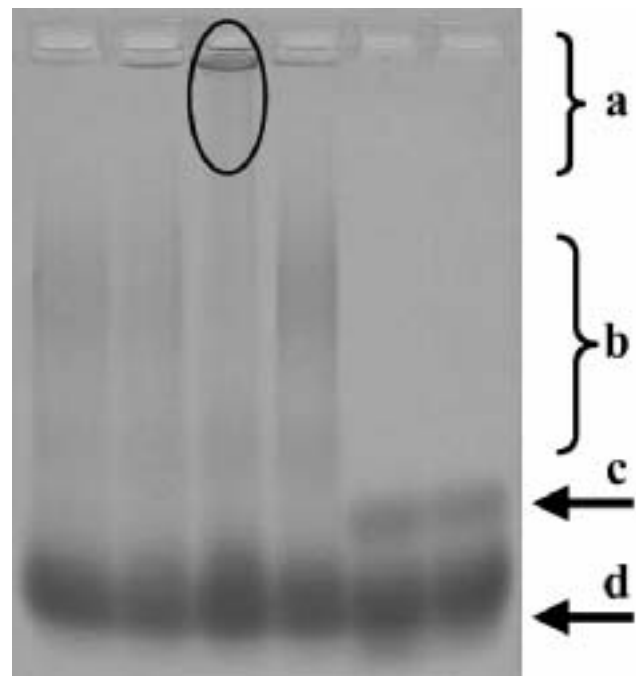

Fig. 5. SDS-agarose gel electrophoresis (0.7%) of heated reconstituted skim milk. All samples were dissolved in a SDS-containing buffer in order to break up physical interactions between the proteins. From left to right the lanes contain milk before gelation; −NEM (lane 1), +NEM (lane 2), after gelation; −NEM (lane 3), +NEM (lane 4), and after gelation + treatment with DTT after dissolving in SDS-buffer; −NEM (lane 5) and +NEM (lane 6). The arrows indicate the structures described in the Results and discussion section (SDS-agarose gel electrophoresis). The circle highlights covalently linked proteins formed during acidification.

of DTT resulted in the formation of a faster migrating protein band (arrow c in lanes 5 and 6). All samples contain a fast migrating protein band (arrow d);

however the differences were observed in the slower migrating region. The protein band indicated by arrow b is only observed in heated milk.

Due to the presence of SDS non-covalent interactions are broken and only covalently bound structures are analyzed. The casein micelles dissociate under these conditions resulting in a fast migrating protein band representing casein monomers and native whey proteins. The slower migrating band (bracket b) in lanes 1 and 2 corresponds to a complex mixture consisting of disulfide-linked casein monomers (oligomers of κ- and αs_2-casein), complexes of cysteine-containing caseins and whey proteins and of whey protein aggregates (Anema & Klostermeyer, 1997; Corredig & Dalgleish, 1996; Jang & Swaisgood, 1990; Oldfield et al., 2000; Rasmussen & Petersen, 1991; Rasmussen, Højrup, & Petersen, 1994; Singh, 1993; Vasbinder, unpublished results). This band is not observed after SDS-agarose gel electrophoresis of unheated milk (results not shown). After gelation disulfide cross-linked structures are formed with a lower migration velocity, which hardly enter the agarose gel. Blocking of thiol groups prevented formation of these larger structures. The reduction of disulfide bonds by DTT resulted in small-sized protein material, probably corresponding to casein and whey protein monomers. In this case, the disulfide-linked structures formed during heat treatment between casein and whey proteins are also reduced. The application of both NEM and DTT clearly demonstrates the formation of disulfide-linked protein structures during acid-induced gelation of heated milk.

3.7.2. Dynamic light scattering

A second method to observe the formation of disulfide-linked structures during gelation is through application of enzymatically cross-linked reconstituted skim milk. Cross-linking of the casein micelles with transglutaminase prevents dissociation in the SDS-buffer needed to dissolve the acid-milk gels (Vasbinder, in press). These modified casein micelles are used as a tool to determine disulfide-linked structures by light scattering. To inactivate the enzyme transglutaminase, milk has to be heated for 25 min at 90°C. The number of exposed thiol groups decreased compared to reconstituted skim milk heated for 10 min (0.07–0.05 mM), but was similar to reconstituted skim milk heated for 25 min. The effect of NEM on the relative decrease in hardness of the gel prepared from transglutaminase-treated milk was comparable to normal treated skim milk, indicating that the enzymic modification does not affect the formation of free thiol groups nor the gel hardness after acidification.

Fig. 6 shows the effect of gelation on the size of particles in milk before and after gelation in the absence and presence of NEM. Before gelation, the modification of the thiol groups had no effect on the size of the
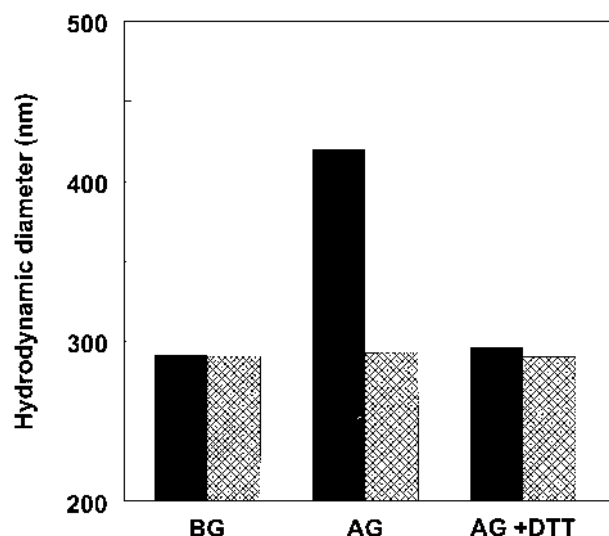

Fig. 6. Size determination by light scattering of control (hatched bars) and blocked (closed bars) samples of the intra-micellar cross-linked micelles, before gelation (BG), after gelation (AG) and after gelation + treatment with DTT (AG + DTT). Measurements were done in duplicate with an experimental error lower than 10%.

particles in transglutaminase cross-linked skim milk. In both cases, particles with a diameter of around 280 nm were determined. After gelation, only in the case of the heated milk sample without NEM treatment were particles with an increased diameter of 430 nm determined. The size of the particles could be decreased to its original value by the addition of DTT, an agent known to break up disulfide bonds.

The observed decrease in the number of thiol groups after prolonged heating, necessary to inactivate the enzyme transglutaminase, is probably due to oxidation of the thiol groups (Hashizume & Sato, 1988; Kirchmeier et al., 1984, 1985; Pofahl & Vakaleris, 1968), as in equally heated reconstituted skim milk the same decrease was determined. The size of transglutaminase cross-linked micelles observed is slightly larger than previously determined (De Kruif, 1997), which can be explained by the presence of SDS. Therefore, we can exclude the occurrence of inter-micellar cross-linking. Ultracentrifugation experiments under non-dissociating conditions revealed that hardly any serum casein was released (Vasbinder, in press). SDS-agarose electrophoresis under dissociating conditions showed that the integrity of the micelle was retained (results not shown). A milk system was obtained which behaved identically to reconstituted skim milk, except that the casein micelles were no longer disrupted in SDS buffer. The light scattering results strengthen the previous results obtained by SDS-agarose gel electrophoresis, where disulfide-linked structures formed during acid-induced gelation at 20°C were visualized.

3.8. General discussion

In our two-stage approach, we distinguish between heat treatment of milk and subsequent acid-induced gelation at 20°C. Whey protein denaturation as occurs in the first step is generally recognized as a very important parameter for the textural properties of yoghurt and other acidified milk products. This is attributed to the altered physical properties of casein micelles due to heating and concomitant coating with whey proteins. The contribution of disulfide formation during the second step, i.e. acidification at ambient temperature, on the textural properties of acid-milk products is hardly recognized in the literature.

In this study, we demonstrated that the formation of disulfide bonds during and after acidification of heated milk contributes strongly to the mechanical properties. The cross-linking process continued even after complete hydrolysis of GDL, while the process levelled off at this point in the presence of NEM. The acidification profile of heated milk, the start of the gelation and the acid-induced microstructure of heated milk observed with CSLM (μm length scale) were not affected by the modification of the thiol groups. Therefore, we suggest that the increase in gel hardness and storage modulus was attributed to the additional disulfide cross-links formed, rather than to changes in the mechanism of aggregation and changes in the microstructure. Moreover, the gel hardness depended on the amount of reactive thiol groups present after heating the milk (heated fresh milk compared to heated reconstituted milk). As it is generally known that acidic conditions are not favorable for the formation of thiol-disulfide bond exchange reactions, it is surprising that these phenomena contribute to such a large extent to the texture of yoghurt. As stated recently by Alting et al. (2000) for acid-induced cold-set gels of whey proteins, the cross-linking was attributed to an increased protein concentration in the protein network formed after diminishing of the electrostatic repulsion near the iso-electric point of the proteins. As far as we know this is the first time that formation of disulfide cross-linked structures during acid-induced gelation of heated milk at ambient temperatures has been demonstrated. Since these processes take place at ambient temperature and on a time scale of hours this finding is highly relevant for the textural properties of acid-milk products, like yoghurt. In fact this knowledge should allow us to control texture.

Acknowledgements

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Chapter 9

Summary and concluding remarks

Summary and concluding remarks

Texture is one of the most important attributes of food products. The perception of texture during consumption is determined by the size and the spatial arrangement of the structural elements of food, their mechanical properties and their response to the dynamic conditions during consumption. These structural elements or so-called meso-structures (size range 50 nm-1 mm) arise from the molecular interactions between individual proteins, lipids and polysaccharides. It is necessary to know how the structures and properties at the meso-level are related to the molecular interactions of food biopolymers, and how the interplay of molecules is affected by the manufacturing process to be able to predict, control and improve the sensory quality of new and existing food products. This thesis deals with cold gelation, an alternative pathway for the efficient use of heat-sensitive proteins as texturizers in food. The purpose of the research described in this thesis was to investigate the molecular mechanism of this gelation process.

Acid-induced cold gelation of globular proteins

The mechanism of cold gelation was reported some 20 years ago. In recent years it was recognized as a novel method to produce protein-based gels. In contrast to heat-induced gelation, cold gelation is a two-step process in which first thermally induced protein aggregates are prepared by a heat treatment at low ionic strength and at a pH far from the iso-electric point of the proteins. The first step of the cold gelation process results in the formation of a stable dispersion of protein aggregates. In the second step gelation is induced by shifting the pH towards the iso-electric point of the protein. Depending on the type of protein and the properties of the aggregates prepared in the first step, cold-set gels can be produced varying in gel properties such as transparency/turbidity, hardness and physical stability.

The unique feature of the cold gelation process is that the (heat-induced) activation step of the proteins (denaturation) is uncoupled from the subsequent gelation process. In the experiments described in this thesis advantage was taken of the possibility to determine and modify the aggregate properties before gelation was induced. Chapter 2 describes the effect of the number of thiol groups exposed at the surface of the aggregates on structural and rheological properties of the resulting gel. The effect of variations in the net electric charge on these properties is discussed in chapter 3. Heating whey protein isolate (WPI) or ovalbumin solutions at different initial protein concentrations enabled us to produce aggregates varying in size and shape. The effects thereof on final gel properties are discussed in chapters 4 and 6. The effect of variation of the protein concentration in the second step of the cold gelation process is described in chapter 5. General and protein-specific mechanisms in the cold gelation process are discussed in chapter 6. Chapters 7 and 8 describe applications of the knowledge obtained in the previous chapters.

Formation of disulfide bonds under acid conditions

At the start of this project it was unclear whether formation of disulfide bonds was still possible under acid conditions. It is well known that disulfide bonds (S-S) and thiol groups (SH) play an important role in the heat-induced gelation of globular proteins and that the strength or rigidity of the resulting gels is related to the number of intermolecular cross-links formed in the heat-induced gel network (Wang and Damodaran, 1990). Formation of disulfide bonds can occur via oxidation of thiol groups or by thiol-disulfide exchange reactions, with a preference for the latter reaction, but both reactions proceed optimally under alkaline conditions.

Our initial experiments were aimed at clarifing if disulfide bonds can still be formed during the acid-induced second step of the cold gelation process. To solve this problem we studied the size of the aggregates after the acidification step as a function of the amount of available thiols on the

surface of the aggregates exposed after the initial heating step. A method was developed to block these surface-exposed thiols (Roefs and de Kruif, 1994) by chemical modification and we optimized the use of agarose gel electrophoresis to allow us to rapidly identify changes in the size of large protein aggregates. Both methods proved to be extremely useful for this study. After solubilization of the cold-set gels in SDS-containing buffer, formation of large, covalently linked aggregates (hydrodynamic diameter >250 nm) could be determined both by SDS-agarose electrophoresis and by dynamic light scattering. The formation of disulfide bonds during the acid-induced gelation step was completely prevented by blocking of the thiol groups on the surface of the WPI aggregates. We clearly demonstrated that under acid conditions disulfide bonds can be formed.
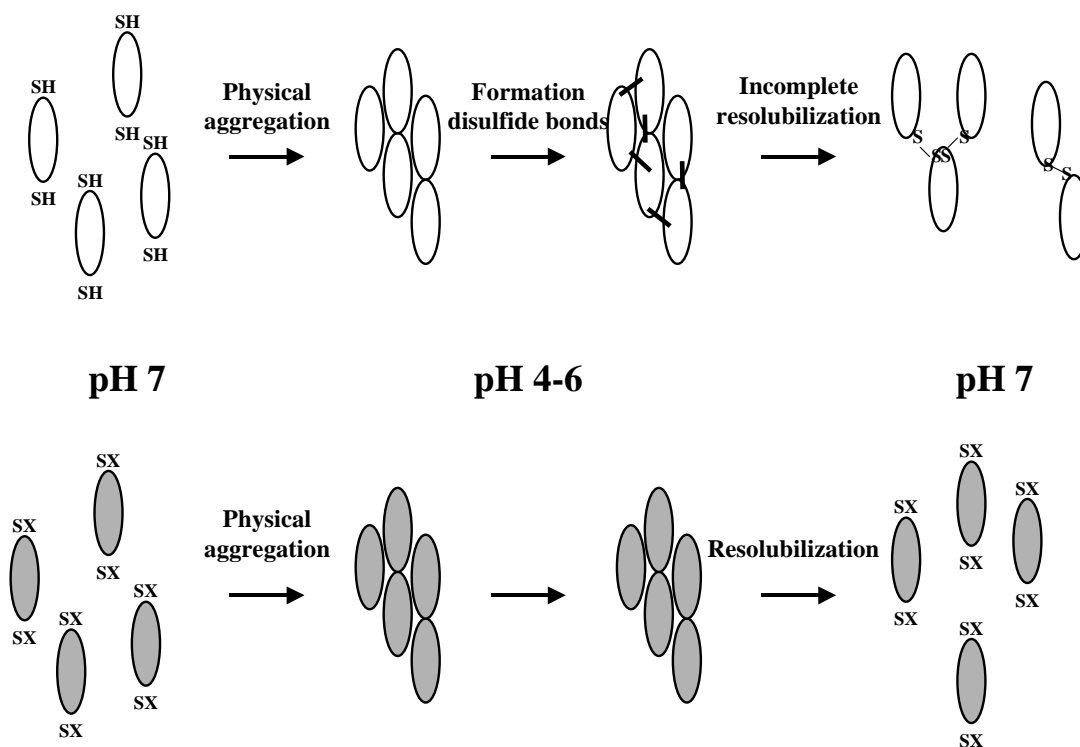
Figure 1:   Model for the formation of intermolecular disulfide bridges and their role during the acid-induced cold gelation of heat-treated whey proteins.

Much to our surprise, microstructural analysis did not reveal any differences in gels prepared from non-modified or thiol-blocked whey protein aggregates. However, gel hardness was significantly decreased (by 10-fold) in gels prepared from thiol-blocked aggregates. This allowed us to put forward a relatively simple model in which we postulated that the initial microstructure of the gels is primarily determined by noncovalent interactions. The additional covalent disulfide bonds were only formed after gelation, probably via thiol-disulfide exchange reactions, and involved in stabilizing the network (preventing of spontaneous rupture and syneresis) and increasing the gel rigidity (**Figure 1**). The unexpected formation of these disulfide bonds under acid conditions was attributed to a large increase of the effective local concentration during the gel state. Probably, as a result of the noncovalent interactions the free thiol groups and the disulfide bonds were very close and formation of disulfide bonds was promoted. As predicted by the model, mixing different amounts of blocked and unblocked aggregates enabled us to control gel hardness without changing the microstructure (on the micrometer length scale). This result could be important for industrial applications where precise control of the hardness of a food product is required.

The net charge

Having resolved the role of thiols, a next question arising from the proposed model was whether the reduction of the electrostatic repulsion of the aggregates (net electric charge) is the origin of the pH-induced gelation. The contribution of electrostatic interactions to the gel properties was previously demonstrated for other types of protein gels by the addition of salt to screen the charge of the protein or by the pH-dependency of gelation characteristics. However, since salts may also have an effect on the hydrophobicity, experiments of this kind do not directly establish the importance of electrostatic interactions (Otte et al., 2000).

Again our approach was to take advantage of the possibility to chemically change the properties of the protein aggregates before gelation was induced. This time, the net-charge of β-lactoglobulin aggregates was modified chemically, either by succinylation of the primary amino groups or by methylation of the carboxylic acid groups. A pure protein fraction was used to better predict changes in the iso-electric point. Modification of the net charge of the aggregates enabled a decrease as well as an increase in the pH regions of gelation as compared to the non-modified aggregates. This allowed the formation of pH-induced gels in the region from pH 2.5 to 9, as compared to pH 4 to 5 for the non-modified aggregates. The shifts in the gelation regions of the succinylated aggregates were in good agreement with the calculated shifts in iso-electric point of the modified aggregates. As far as we know, this is the first time that the importance of the net electric charge has been demonstrated by intrinsic modifications at the protein molecular level and not by changing the system conditions. In addition, we demonstrated that the same principles hold for WPI as for purified β-lg.

We also demonstrated that the formation of additional disulfide bonds was affected by the iso-electric point of the protein and that this had a clear effect on the properties of the gel. Characterization of the gels prepared from aggregates having the highest degree of succinylation (gelation at pH 2.5-3.5) revealed that disulfide bonds between aggregates were 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 becomes possible via the iso-electric point of the protein aggregates. These results can be of help to industrial applications in which pH of the product is changed or when replacing proteins.

Aggregate size

The third important aggregate characteristic that was to expected to affect final gel properties is the size of the aggregates. Ju and Kilara (1998) already reported a clear effect of the aggregate size on the large deformation properties of cold-set WPI gels. Application of thiol-blocked WPI aggregates enabled us to distinguish between the effect of structural and chemical properties of the aggregates.

Protein aggregates with clearly different structural (size, intrinsic viscosity) and chemical characteristics (number of thiol groups) were prepared by variation of the protein concentration at heating. Heating conditions were chosen such that differences in the degree of aggregation were excluded. Although gel hardness indeed seemed to correlate with the different structural aggregate features as reported before in the literature (Ju and Kilara, 1998), the differences in hardness could for the most part be eliminated by blocking of the thiol groups. If the thiol groups present on the surface of the WPI aggregates were chemically blocked, only a minor effect of the difference in structural features, such as hydrodynamic diameter and intrinsic viscosity, on the gel hardness remained. Formation of larger disulfide cross-linked protein structures paralleled the increase in gel hardness. In addition, the effect of the presence of native non-aggregated protein on the final gel properties can be excluded, since in our gelation experiments most protein (> 95%) participated in the formation of a protein network. Therefore, in contrast to Ju and Kilara (1998), we concluded that the hardness of cold-set WPI gels was determined by the number of thiol groups rather than by

the size of the aggregates or other structural features. The results emphasize once more the importance of disulfide bond formation for industrial applications.

Scaling behavior of structural and rheological properties

In the literature different models exist that describe the scaling behavior of gel properties with the protein concentration. The concept of fractals (Mandelbrot, 1984) provides an elegant and useful approach to explain the scaling behavior of the structural properties of the aggregate network. In this model the geometric structure is assumed to be scale-invariant. A single parameter D, called fractal dimensionality, describes the geometric properties of the network for the typical length scales at which the random aggregation mechanism occurs. Several theoretical models have been developed to predict the scaling behavior of micro-structural properties and rheological properties with the protein concentration on the basis of the fractal dimension, in both heat- and cold-set gels (Shih et al., 1990; Bremer, 1992; Mellema, 2001; Wu et al., 2001). It was however clearly demonstrated by Verheul and Roefs (1998) that for heat-set gels of WPI only a fraction of the protein is aggregated and available for the building of a protein network at the point when the initial microstructure is formed. In contrast to heat-induced gelation, with acid-induced cold gelation all the protein is in its aggregated form and available to participate in the protein network from the start of the gelation. Therefore it was expected that cold-set gels would better satisfy the conditions for fractal aggregation than heat-set gels do.

Two different gel systems were used: gels made from WPI aggregates with and without thiol-blocking treatment, to identify the contribution of covalent and noncovalent interactions to the scaling behavior of the structural and rheological properties in a cold-gelling protein system. Analysis of the structural characteristics of acid-induced gels of both thiol-blocked and unmodified whey protein aggregates yielded a fractal dimension (2.3 ± 0.1) which is in line with results for other comparable protein networks. However, application of known fractal scaling equations to our rheological data yielded ambiguous results. Our results suggest that acid-induced cold gelation probably starts off as a fractal process, but is rapidly taken over by another mechanism at larger length scales (> 100 nm), such as spinodal decomposition. In addition, indications were found for the occurrence of disulfide cross-link-dependent structural rearrangements at smaller length scales (< 100 nm). Due to all these rearrangements the fractal concept cannot be applied here.

The results of the permeability experiments were also compared with the work of Verheul and Roefs (1998 a,b). In their studies heat-induced gels were prepared from WPI at the same heating temperature as applied in our first step of the cold-gelation process, but at other pH values and at higher concentration of salt. By application of the cold-gelation method, gels can be prepared with the same permeability characteristics but at a much lower protein concentration than with heat-induced gelation (respectively 0.5% and 3%). Thus, cold gelation offers the food industry a more efficient use of protein.

General implications for globular protein gelation mechanisms

To extend the scope of our findings we compared the cold gelation of ovalbumin with that of WPI. We selected ovalbumin, in particular since the shape of aggregates prepared from this globular protein clearly differed from those prepared from WPI. Under the heating conditions applied in the first step of the cold gelation process ovalbumin forms soluble fibrillar aggregates (Koseki et al., 1989; Weijers et al., 2002).

To corroborate the importance of thiol groups for the cold gelation of ovalbumin aggregates, those conditions were determined for ovalbumin that resulted in the formation of aggregates that consisted of disulfide cross-linked protein monomers and having reactive thiol groups exposed at their surface. The WPI aggregates had a curved strand-like morphology and the ovalbumin aggregates were clearly fibrillar (Figure 2). During acid-induced gelation, when the pH decreased towards the iso-electric point of the proteins, the characteristic ordering (observed as a typical inter-

particle distance in the nm range using SAXS), caused by the repulsive character of both types of aggregates, disappeared and was replaced by a random distribution of uncharged aggregates. These processes were not affected by differences in size and shape and can therefore be expected to occur in a similar fashion for any type of protein aggregate that is acidified towards its iso-electric point.
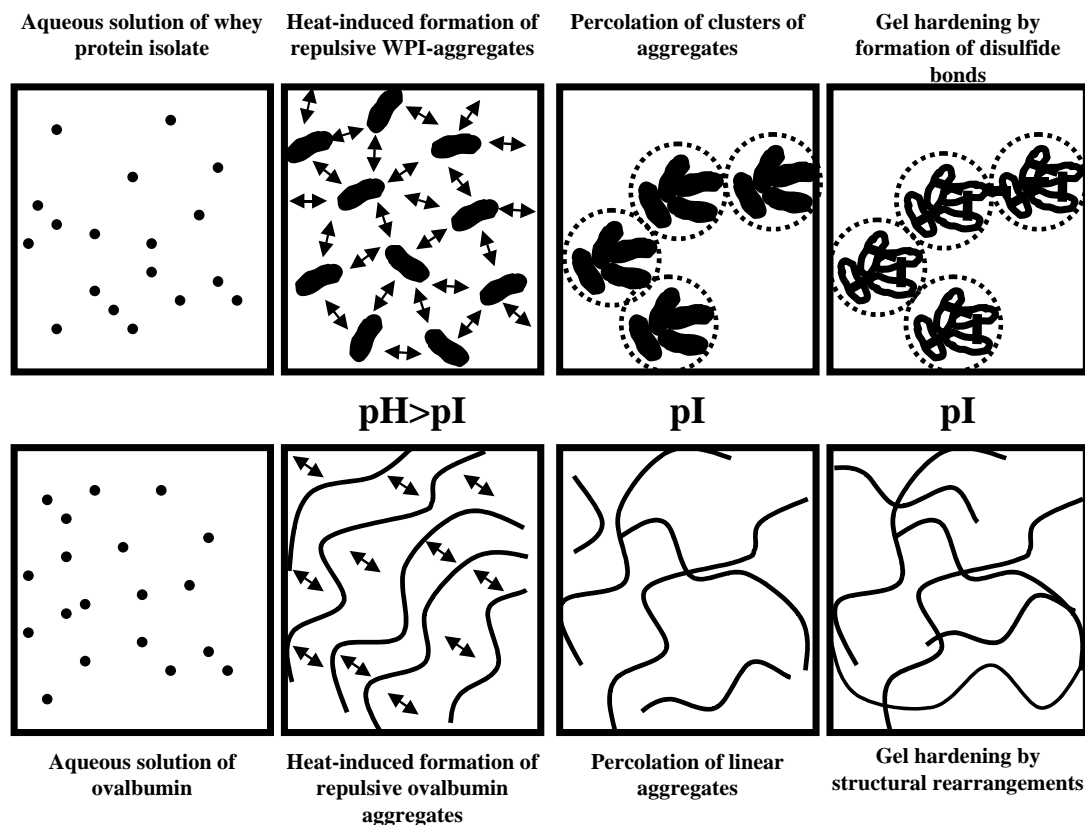
Figure 2: Schematic representation of the acid-induced cold gelation process of WPI (upper panels) and ovalbumin (lower panels).

Differences in size and shape of protein aggregates were found to influence small deformation properties and the appearance of the cold-set protein gels. Longer fibrillar aggregates (ovalbumin) have a lower overlap concentration, which causes gelation to commence earlier and results in more effective contact points, yielding higher G' values and more transparent gels (**Figure 2**). Smaller linear aggregates (WPI) first form clusters that subsequently percolate, yielding more turbid gels and lower G' values.

Although for WPI (thiol-blocked) almost no effect of the size on gel hardness was found, for ovalbumin a large effect of the size, but more probably of the shape on the gel hardness was found. The formation of additional disulfide bonds during gelation was only found for WPI and seems to depend not only on the number of thiol groups, but also on the number and accessibility of disulfide bonds in the protein used. The presence of thiol groups and/or disulfide bonds in the native protein did not guarantee the formation of stabilizing disulfide bonds after gelation. As previously reported for heat-induced protein gels (Matsudomi et al., 1992; Legowo et al., 1993, 1996; Rojas et al., 1997) and pressure-induced proteins gels (Ipsen et al., 2002), the presence of α-lactalbumin, a protein containing four cystines (S-S), has a positive effect on the gel hardness of acid-induced cold-set gels probably by enhancing the number of thiol-disulfide exchange reactions. As commercial protein preparations are mixtures of proteins, this effect of α-lactalbumin demonstrates

a possible mechanism by which the composition of a protein preparation can influence final textural properties.

Cold gelation induced by bacterial acidification

As the formation of a protein network and the formation of disulfide bonds are both time- and pH-dependent, it was expected that they can be controlled by the acidification rate and depend on the final pH reached after acidification. Therefore, we applied acid-producing bacteria to control the acidification process by varying inoculum size and glucose concentration (carbon source). In addition, the use of bacterial instead of chemical (glucono-δ-lactone) acidification in the process of cold gelation is of practical relevance, because microbial acidification is often preferred to "food-grade"chemical acidification.

Gel hardness of bacterially acidified cold-set protein gels of ovalbumin and WPI could be adjusted by the final pH and by the time of acidification. The pH dependency of the gel hardness is probably related to the net charge of the protein aggregates. Depending on the type of protein, the time-dependent formation of disulfide bonds contributes to the textural properties of cold-set protein gels. Based on the results a patent application was filed.

Disulfide formation in acid-induced milk gels

Since we learned from our research on the acid-induced cold gelation process that formation of disulfide bonds is possible under acidified conditions, we applied this knowledge to verify the importance of formation of these bonds in acidified milk products (yogurt). Denaturation of whey proteins during pasteurization of milk results in the formation of whey protein aggregates and in the coating of the casein micelles with whey proteins (Vasbinder et al., 2003). Clearly, heated milk shows similarities with a dispersion of WPI aggregates. A substantial number of thiol groups remained exposed after cooling.

Analogous to the cold gelation process of globular proteins, we demonstrated by the use of agarose electrophoresis that larger disulfide-linked protein structures were formed during acidification of heated milk at ambient temperature. By using transglutaminase cross-linked casein micelles (no dissociation in the SDS-buffer) as a tool we were able to confirm the electrophoresis results by light scattering. To our knowledge, this is the first time that formation of disulfide-cross-linked structures during acid-induced gelation of heated milk at ambient temperatures has been demonstrated.

The time-dependent formation of the disulfide-cross-linked structures contributed significantly to the mechanical properties of acid milk gels, resulting in gels with an increased storage modulus and hardness, making addition of WPI aggregates a viable method to thicken acid milk products like yogurt (Britten and Giroux, 2001). Addition of a thiol-blocking agent prevented the formation of disulfide-linked structures. The mechanical properties were shown to be the result of the contribution of denatured whey proteins to the protein network as such and the additional formation of disulfide bonds. Since these thiol-disulfide exchange reactions take place at ambient temperature and under acidic conditions, the disulfide cross-linking is highly relevant for textural properties of acid-milk products, like yogurt.

Concluding remarks

The model for the acid-induced cold gelation of cysteine-containing globular proteins postulated at the beginning of this study (Figure 1) was confirmed and extended in the course of this study (Figure 2). In the first step of the cold gelation process repulsive linear-shaped protein aggregates were prepared by heating. Reduction of the electrostatic repulsion, as a result of the acidification, initiated the formation of a randomly organized protein network by physical interactions. This process was not affected by the type of protein, by variations in size and shape, or by the ability to form disulfide cross-links. Therefore, it is expected that this part of the cold

gelation process will occur in a similar fashion for any type of globular protein aggregate that is acidified towards its iso-electric point. The cold gelation process makes more efficient use of the protein than heat-induced gelation, since most of the protein directly contributes to the building of the protein network. Additional formation of disulfide bonds during gelation contributes to gel strength and stabilizes the protein network (physical stability), preventing spontaneous gel rupture and syneresis (expulsion of water). The formation of disulfide bonds depends on the number and accessibility of thiol groups and disulfide bonds present in the protein molecule in question. Therefore, these are important control parameters that can be used to tune the texture of (cold-set) gels. In addition, the contour length of the linear-shaped aggregates prepared in the first step also clearly affects the gel strength by determining the moment at which percolation occurs. Either direct percolation of relatively long fibrillar protein aggregates occurs or formation of clusters of smaller aggregates precedes percolation. Moreover the length of the linear-shaped aggregates determines the appearance of cold-set gels.

This work has shown that the process of cold gelation is applicable for soluble globular proteins. The information obtained can be translated to other proteins, since the characteristics of the protein aggregates prepared in the first step of the cold gelation process, such as number and accessibility of thiol groups and disulfide bonds, length and net charge, determine most of the final gel properties after acid-induced gelation.

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Samenvatting

Textuur speelt een centrale rol in de sensorische eigenschappen van voedingsproducten. Het is één van de belangrijkste criteria voor consumenten om een product te beoordelen op kwaliteit en versheid. Dit proefschrift behandelt een alternatieve methode voor het efficiënt gebruik van hitte-gevoelige eiwitten als "texturizer" in voedingsmiddelen: de koude gelering van globulaire eiwitten. Het doel van het onderzoek beschreven in dit proefschrift was het ophelderen van de moleculaire mechanismen van deze geleringmethode.

Hoofdstuk 1 geeft een kort overzicht van de gelering van globulaire eiwitten in het algemeen en van koude gelering in het bijzonder. Koude gelering is gedefinieerd als het geleringproces van eiwitten bij relatief lage temperatuur (kamertemperatuur). Voor dit proces wordt een dispersie van geactiveerde eiwitdeeltjes gebruikt. Gelering kan worden geïnduceerd door de toevoeging van zout, een verandering van de pH of de toevoeging van een specifiek enzym of chemische crosslinker. Strikt genomen vallen ook het kaas- en yoghurt-bereidingsproces hieronder, maar in de literatuur wordt de term koude gelering beperkt tot de gelering van niet-caseïne eiwitten. Omdat gelering kan worden geïnduceerd op een later tijdstip in de productie en zelfs in de verpakking, heeft deze methode interessante toepassingen bij het textureren van voedingsproducten, het maskeren van aroma's en het encapsuleren van specifieke componenten.

Koude gelering onderscheidt zich van het veel toegepaste hitte-geïnduceerde geleringproces van globulaire eiwitten, omdat het een twee-staps proces is. In tegenstelling tot hitte-geïnduceerde gelering zijn bij koude gelering de denaturatie- en aggregatieprocessen duidelijk gescheiden van het geleringproces. Hierdoor ontstaat een unieke mogelijkheid om, voordat gelering wordt geïnduceerd, de eigenschappen van eiwitaggregaten te bepalen en te modificeren. Dit voordeel is systematisch toegepast om te bestuderen hoe verschillende aggregaateigenschappen van cysteïne-bevattende globulaire eiwitten (wei-eiwit isolaat (WPI), β-lactoglobuline en ovalbumine) de uiteindelijke eigenschappen van de resulterende zuur-geïnduceerde koude gelen bepalen en hoe covalente en niet-covalente bindingen hieraan bijdragen.

Hoofdstuk 2 beschrijft het eerste onderzoek naar de rol van (covalente) disulfide bindingen in het koude geleringproces. Thiol-groepen, geëxposeerd tijdens de bereiding van de aggregaten in de eerste stap van het geleringproces, werden geblokkeerd middels een chemische modificatie-reactie. Met behulp van deze gemodificeerde aggregaten kon worden aangetoond dat tijdens de verzuring naar het iso-electrische punt van de eiwitaggregaten, een initieel eiwitnetwerk wordt gevormd door fysische interacties. Verrassend was dat kon worden aangetoond, dat onder zure condities, dit netwerk vervolgens werd gestabiliseerd door de vorming van covalente disulfide bindingen, resulterende in sterkere en fysisch stabielere gelen. De vorming van disulfide bindingen onder zure omstandigheden kon worden verklaard door de lokaal hoge eiwitconcentratie in een eiwitnetwerk.

Hoofdstuk 3 beschrijft hoe middels chemische modificatie van de netto-lading van wei-eiwit-aggregaten werd veranderd en hoe vervolgens de bijdrage van elektrostatische interacties aan het koude geleringproces kon worden vastgesteld. Deze studie toonde duidelijk aan dat reductie van de elektrostatische interacties de drijvende kracht is achter het koude geleringproces en dat vorming van disulfide bindingen onder zure omstandigheden nog plaats kan vinden tot een pH van 3.5.

Het eerder gerapporteerde effect van de aggregaatgrootte op de stevigheid van cold-set gelen is nader onderzocht in **hoofdstuk 4**. Aggregaten van verschillende groottes werden bereid door verhitting bij verschillende eiwitconcentraties. Door vervolgens de geproduceerde koude gelen te vergelijken met die bereid van thiol-geblokkeerde aggregaten kon onderscheid worden gemaakt tussen structurele en chemische aggregaateigenschappen. Uit dit werk werd geconcludeerd dat het effect van de hoeveelheid thiolgroepen aanwezig op de oppervlakte van de aggregaten domineert over het effect van de grootte op de uiteindelijke gelsterkte van koude wei-eiwitgelen.

Hoofdstuk 5 beschrijft het schalingsgedrag van structurele en rheologische eigenschappen van cold-set gelen met de eiwitconcentratie. Op basis van de resultaten van dit hoofdstuk werd gesuggereerd dat zuur-geïnduceerde gelering van start gaat als een random-aggregatieproces, maar wordt overgenomen door een fasescheidingsproces. Daarnaast werden aanwijzingen gevonden voor disulfide afhankelijke structurele herrangschikkingen, die optreden na de vorming van het gel.

In **hoofdstuk 6** werd de koude gelering van wei-eiwit vergeleken met die van ovalbumine. Eiwitspecifieke en meer generieke mechanismen konden hierdoor worden onderscheiden. Voor beide eiwitten werd vastgesteld dat de karakteristieke ordening veroorzaakt door het repulsieve karakter van de eiwitaggregaten verdween tijdens verzuring naar het iso-electrische punt van de eiwitten en werd vervangen door een willekeurige verdeling. Dit proces was niet afhankelijk van het type eiwit-aggregaat en van eigenschappen als grootte, vorm en concentratie thiolgroepen.

De al eerder aangetoonde vorming van disulfide bindingen bepaalde in hoge mate de stevigheid van de koude gelen. De vorming van deze bindingen bleek af te hangen van de concentratie thiolgroepen, maar ook van de hoeveelheid en toegankelijkheid van de cystines in het eiwitmolecuul. Het belang van de vorm van de aggregaten werd gedemonstreerd door de significante bijdrage van fibrillaire structuren aan de gelsterkte.

Hoofdstuk 7 en **8** beschrijven hoe de kennis opgedaan in de eerste hoofdstukken van dit proefschrift konden worden toegepast in microbieel verzuurde koude gelen en in chemisch verzuurde verhitte melk (yoghurt-model). De stevigheid van microbieel verzuurde gelen bleek naast de eind-pH, met name afhankelijk van de tijd dat het systeem in de geltoestand was. Ook in chemisch verzuurde melk kon worden aangetoond dat in de tijd de eiwitstructuur werd versterkt door de vorming van disulfide bruggen.

Hoofdstuk 9 geeft een samenvatting van de belangrijkste resultaten en conclusies.

Nawoord

Het is af! Na 4 jaar komt er een einde aan mijn WCFS-NIZO dubbelleven. Alhoewel ik geen "standaard"-AIO tijd heb beleefd kan ik toch terugkijken op een leerzame en plezierige periode.

Onderzoek doe je niet alleen! Verschillende mensen hebben bijgedragen aan de totstandkoming van dit proefschrift. Allereerst, Herma bedankt voor je vertrouwen, steun en geduld. Het promotieonderzoek vond plaats in een drukke periode van ons leven. We hebben ons huis verbouwd en zijn de trotse ouders geworden van 2 mooie dochters.

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Het initiatief van de NIZO-AIO's om een reguliere bespreking te houden werd door Kees overgenomen en in navolging van ABBA en K3 vernoemd naar de eerste letters van de namen van de AIO's, AAFJE (Astrid, Arno. Fanny en Joanke). Alhoewel ik deze bespreking soms wel eens vergat heb ik de discussies met de collega-AIO's zeer gewaardeerd.

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Ook wil de andere (ex-)collegae van NIZO food research en WCFS bedanken voor de collegiale werksfeer. De directies van NIZO food research en WCFS wil ik bedanken voor de geboden gelegenheid om 4 jaar promotieonderzoek te mogen verrichten.

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Arno

Curriculum Vitae

Aart Cornelis (Arno) Alting werd geboren op 26 oktober 1964 in Oegstgeest. In 1984 behaalde hij het VWO diploma aan het Christelijke Streeklyceum te Ede. Hierna begon hij met de studie HLO chemie aan de Rijks Hogere Agrarische School "Wageningen" (tegenwoordig Hogeschool Larenstein, Velp). Eind 1988 behaalde hij zijn diploma en in 1989 begon hij als wetenschappelijk assistent bij het Nederlands Instituut voor Zuivelonderzoek (NIZO). In datzelfde jaar werd ook begonnen met een deeltijdstudie Biologie aan de Universiteit van Utrecht, welke in 1994 werd afgerond met als hoofdvak moleculaire biologie. Na het behalen van dit diploma werd hij wetenschappelijk medewerker bij NIZO food research. Op de afdeling Biofysische Chemie werkte hij achtereenvolgens aan de karakterisering van het proteolytisch systeem van de melkzuurbacterie *Lactococcus lactis*, de aan aromavorming gerelateerde afbraak van eiwitten en aminozuren in kaas en aan de hydrolyse en de antigeniciteit van melkeiwitten. Op de afdeling Product Technology werd zowel gewerkt aan de hydrolyse van (melk)eiwitten, de productie van bio-actieve peptiden, als aan de koppeling van (melk)eiwitten, hitte-geïnduceerde aggregatie en enzymatische cross-linking. In 1998 werd hij bestuurslid van het Genootschap ter bevordering van Melkkunde, voor een periode van 4 jaar, waarvan 3 jaar als secretaris. Van mei 1999 tot mei 2003 werd hij voor 70% gedetacheerd naar het Wageningen Centre voor Food Sciences, waar het in dit proefschrift beschreven onderzoek werd verricht. Hij is (co)auteur van meer dan 35 wetenschappelijke publicaties, 1 patent en 1 patent aanvraag. Hij is getrouwd met Herma. Samen hebben ze twee dochters, Imara (3) en Farah (1).

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