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Modulation of the Gelation Efficiency of Fibrillar and Spherical Aggregates by Means of Thiolation

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Supporting Information

ABSTRACT: Fibrillar and spherical aggregates were prepared from whey protein isolate (WPI). These aggregates were thiolated to a substantial degree to observe any impact on functionality. Sulfur-containing groups were introduced on these aggregates which could be converted to thiol groups by deblocking. Changes on a molecular and microstructural level were studied using tryptophan fluorescence, transmission electron microscopy, and particle size analysis. The average size (nm) of spherical aggregates increased from 38 to 68 nm (blocked variant) and 106 nm (deblocked variant) after thiolation, whereas the structure of fibrillar aggregates was not affected. Subsequently, gels containing these different aggregates were prepared. Rheological measurements showed that thiolation decreased the gelation concentration and increased gel strength for both WPI fibrillar and spherical aggregates. This effect was more pronounced upon thiolation of preformed fibrillar aggregates. The findings suggest that thiolation at a protein aggregate level is a promising strategy to increase gelation efficiency.

KEYWORDS: *chemical cross-linking, thiolation, whey protein isolate, fibrillar, spherical, aggregates, gelation efficiency*

INTRODUCTION

Whey proteins (WP) are widely used as food ingredients because of their nutritional and functional properties.[1] Past research has yielded valuable information on structural changes in WP as a function of protein concentration, pH, and ionic strength upon exposure to heat.[2−4] The behavior of WP upon heating is of interest because, when well understood, protein denaturation and aggregation events can be directed to result in novel materials[5,6] with diversity in size and functional properties. Micrometer-scale three-dimensional amorphous particles whose internal structure is not strictly spherical, but will be referred to as "spherical" aggregates in this article, are claimed to be used in applications that may vary from thickening agents[7] to carriers for encapsulation,[8] and fat substitutes to improve creaminess.[8] Fibrillar aggregates (fibrils) which are a few nanometers thick, may be used in low-caloric products because of their functionality that ranges from gelling agents to stabilizers of foams and emulsions.[9] Past research on fibrillar[1,10−12] and spherical[13] aggregates has reported their assembly process.

Protein gelation is the result of aggregation in which polymer to solvent interactions are balanced and a network is formed.[14] Both fibrillar and spherical aggregates can be organized into three-dimensional gel networks. The conditions in which the transformation from aggregates to gel networks occurs largely determines visual and rheological properties of the resulting gel networks.[14] Fibrils can be organized into a network by different approaches.[15,16] Apart from these reports,[15,16] limited attention has been given to the formation of gels from preformed fibrils. Turbid, particulate gels are formed by spherical aggregates, for instance, by adding high salt concentrations,[13] by slow acidification toward isoelectric point (IEP) using glucono-δ-lactone,[14] or by heat induced gelation.[13] It is generally found that cold-set gelation of spherical aggregates results in gels with superior strength over heat-induced ones.[13,14]

Sulfhydryl (SH) groups and disulfide bonds (S−S) play a significant role in the formation of protein gels.[17−19] A number of functional properties such as viscosity, texturization, thermal stability, and elasticity have been directly or indirectly linked to sulfhydryl content of proteins.[20] The number as well as reactivity of thiol groups has been reported to have an influence on rheological behavior of proteins.[21] Studies into the role of thiol groups and disulfide bridges in aggregation and gelation processes have been performed by either the blocking of the thiol groups naturally present in the protein molecule[13] or by the introduction of thiol groups (thiolation) using *S*-acetylmercaptosuccinic anhydride (S-AMSA).[22,23] The mechanism of this reaction is shown in Figure 1. The relevance of such modifications for food applications has been recently discussed by de Jongh and Broersen (2012).[24]

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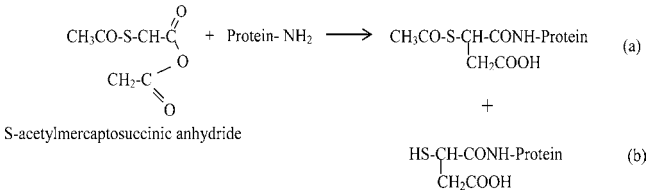

Figure 1. Reaction mechanism of S-AMSA. An acetylthio group is attached to the amines of the protein in a blocked form (a) which can subsequently be deblocked to result in a reactive form of the thiol group (b).

In most studies, native proteins were used as a starting point for the thiolation procedure after which the aggregation and consequently gel formation properties of the protein were evaluated.[21,25] A concomitant impact on gelation was however reported due to the formation of different type of aggregates.[23] In this study, fibrillar and spherical aggregates were prepared from unmodified whey protein isolate (WPI), and subsequently, these structural building blocks were chemically modified by thiolation. The objective of this study was to investigate the influence of additional thiol groups on gel formation, obtained by acidification of WPI fibrillar and spherical aggregates, in order to enhance the mechanistic understanding of the role of additional thiol groups in protein aggregation, interactions, and subsequent gelation behavior. It is hypothesized that more impact can be achieved by thiolation using fibrillar as opposed to spherical aggregates. This might be related to higher excluded volume in fibrillar compared to spherical aggregates at the same protein concentration. Our approach involved the covalent attachment of acetylthiogroups to preformed aggregates. This approach is judged to be beneficial because starting with preformed fibrillar or spherical aggregates optimizes the use of exposed functional groups to undergo direct cross-linking as opposed to folded protein molecules which change conformation multiple times prior to "docking" into aggregates. Second, a high degree of thiolation of natively folded protein has been shown to inhibit the initial aggregation process, which, in turn, greatly affects its ability to induce strong gel formation.[23] Results from rheological characterization of networks from thiolated WPI fibrillar and spherical aggregates will give an insight into the importance of interactions between WP structural building blocks and hence the potential use of these structural building blocks in food applications.

MATERIALS AND METHODS

Materials. Whey protein isolate (WPI) Bipro was obtained from Davisco Food International, Inc. (Le Sueur, MN, USA). Glucono-δ-lactone (GDL), *S*-acetylmercaptosuccinic anhydride (S-AMSA), ortho-phthaldialdehyde (OPA), *N*,*N*-dimethyl-2-mercaptoethyl-ammonium-chloride (DMA), disodiumtetraborate decahydrate (Borax), sodium dodecylsulfate (SDS), uranyl acetate, and hydroxylamine hydrochloride (ReagentPlus, 99%) were all obtained from Sigma-Aldrich (Steinheim, Germany). 2,2 V-Dipyridyldisulfide (PDS) was obtained from Alfa Aesar (Karlsruhe, Germany). Thioflavin T (Th T) was obtained from Acros organics (Geel, Belgium). All reagents were analytical grade and used without further purification.

WPI Fibril Preparation. Protein solutions of 9 wt % WPI were prepared by dispersing WPI powder in Milli-Q water adjusted to pH 2 using HCl, followed by overnight stirring at 300 rpm and 4 °C. The obtained solutions were then centrifuged at 15000*g* for 30 min at 4 °C to remove traces of insoluble material. The supernatant was filtered through a 0.45 μm (Millex-SV, Millipore Corp., Bedford, MA) filter. The final protein concentration was corrected to 2.5 wt % unless stated otherwise. Protein content was determined using DUMAS (NA 2100 Protein, CE instruments, Milan, Italy) with a conversion factor of 6.38.

Fibril preparation was carried out according to a previously described protocol.[1] In summary, 20 mL aliquots of 2.5 wt % protein solutions prepared as described above were incubated at 80 °C for 24 h at pH 2 in a temperature-controlled water bath and continuously stirred at 300 rpm. After the heat treatment, samples were cooled in an ice bath and stored at 4 °C until further use. Th T fluorescence assay was used to evaluate the formation of WPI fibrils as previously described.[9] After 24 h of heating, a volume of 48 μL of heated WPI samples was mixed with 4 mL of filtered (0.2 μm, Schleicher and Schuell) 60 μM Th T solution in phosphate buffer (10 mM phosphate, 150 mM NaCl at pH 7.0), and incubated for 10 min at 25 °C. Fluorescence intensity was measured using a fluorescence spectrophotometer (Perkin-Elmer LS 50 B) at an excitation wavelength of 460 nm (slit width 2.5 nm) and emission wavelength from 465 to 550 nm (slit width 2.5 nm). Controls included the fluorescence intensity of Th T solution without the addition of protein solution and unheated WPI solutions.

WPI Spherical Aggregate Preparation. WPI spherical aggregate solutions were prepared by dispersing 9 wt % WPI powder in Milli-Q water (Millipore Corp., Billerica, MA), followed by stirring at 25 °C, 300 rpm for 2 h. Portions of 100 mL protein solutions at an initial pH 7 were incubated in a water bath at 68.5 °C for 2.5 h. This heat treatment has been shown to result in over 95% aggregation of the proteins.[14] WPI aggregate solutions were then cooled using running tap water for 30 min and subsequently stored overnight at 4 °C.

Determination of hydrodynamic diameters of the protein aggregates was carried out using dynamic light scattering (Zetasizer Nano ZS, Malvern Instruments, Worcestershire, UK). The spherical aggregate solutions at pH 7 were diluted to 0.5 wt % using Milli-Q water and triplicate measurements were carried out at 25 °C. Reported values are the average hydrodynamic diameters of the largest intensity peak as found in the distribution analysis using Malvern DTS 5.03 software.

Thiolation of WPI Structural Building Blocks into Blocked (SX) and Reactive (SH) Variants. Thiolation was carried out on the above derived structural building blocks (fibrillar and spherical aggregates) as previously described.[26] The pH of a 50 mL of a 2.5 wt % WPI fibrillar and spherical aggregates protein solution was adjusted to pH 8 using 6 M NaOH. Aliquots of the solutions were kept separate and used without further treatment to serve as unmodified controls. Optimization of S-AMSA to amines ratio was performed initially with regard to degree of substitution (DS) and reactivity. This led to selection of a ratio of 1:8 (S-AMSA/amines) to obtain medium DS of around 60%.

S-AMSA reagent was added to the protein solutions in aliquots of 5 mg to a total of 97.5 mg while maintaining a constant pH 8.0 ($\pm$0.1). After S-AMSA reagent was added, the solution was stirred for 24 h at room temperature followed by extensive dialysis against distilled water at 4 °C (MWCO 12000−14000 Da dialysis membrane). Dialyzed solutions were separated into two fractions. To the first fraction, a final concentration of 0.01 M hydroxylamine hydrochloride was added to aid in deblocking of thiol groups by removal of the acetyl groups. This sample contained exposed thiol groups (SH). After addition of 0.01 M hydroxylamine hydrochloride, the SH fraction was stirred (25 °C, 1 h, 300 rpm), followed by dialysis against distilled water for 24 h at 4 °C (MWCO 12000−14000 Da dialysis membrane). To the second fraction, no further treatment was carried out, thus yielding a fraction in which thiol groups were present in a "blocked" form, still containing the acetyl group (SX). The samples were subsequently stored at 4 °C and used within 2 weeks. Thiolation carried out in this way resulted in the formation of six WPI variants as presented in Table 1.

Analytical Characterization of WPI Structural Building Blocks. The total number of sulfhydryl groups was determined using Ellman's reagent.[27] The number of primary amines (lysines and the protein N-terminal amine groups) was determined using chromogenic ortho-phthaldialdehyde (OPA) assay as previously described.[28] OPA results were used to determine the degree of substitution (DS) of amines with thiol groups. Apparent chemical

Table 1. Overview of Unmodified and Thiolated (SX-Blocked and SH-Deblocked Variants) WPI Fibrillar and Spherical Aggregates As Used in This Work[a]

	blocked/ deblocked	variant abbreviation
WPI fibrils	unmodified	F
thiolated WPI fibrils	blocked	FSX
thiolated WPI fibrils	deblocked	FSH
spherical WPI aggregates	unmodified	A
thiolated spherical WPI aggregates	blocked	ASX
thiolated spherical WPI aggregates	deblocked	ASH

[a]F is fibrillar aggregates, and A is spherical aggregates.

reactivity of thiol groups was measured for each sample using the sulfhydryl−disulfide exchange index (SEI) as previously described.[29] SEI determines the reactivity of blocked and deblocked protein variants based on the reaction between 2,2V-dipyridyldisulfide (PDS) and free −SH groups.[30] Ellman's, OPA and SEI assays were all carried out in duplicate.

Microscopic and Spectrophotometric Characterization of WPI Structural Building Blocks. Unmodified and thiolated WPI fibrillar and spherical aggregates were prepared for transmission electron microscopy (TEM) by negative staining according to the previously described protocol.[15] In summary, a 10 μL aliquot of 0.025 wt % protein solution was deposited onto a carbon support film on a copper grid (Aurion-Immuno Gold Reagents & Accessories, Wageningen, The Netherlands). The excess protein solution was removed after 30 s using filter paper (Whatman No. 1, 512-1002, VWR International Europe BVBA, Leuven, 3001, Belgium). A droplet of 2% uranyl acetate, pH 3.8, was added for 30 s for improved contrast, and any excess was removed by use of filter paper. Electron micrographs were obtained using a Jeol JEM1011 transmission electron microscope (Tokyo, Japan) operating at 80 kV.

Intrinsic tryptophan fluorescence spectra of 50 μg/mL WPI sample solutions in Milli-Q water were obtained using a Cary eclipse fluorescence spectrophotometer (Agilent technologies, The Netherlands) upon excitation at a wavelength of 280 nm, and the emission spectra were recorded at a wavelength range from 300 to 400 nm at 25 °C. Quartz cuvettes with an optical path of 1 cm were used. The excitation and emission slit widths were 5 nm, and a scan speed of 120 nm/min was used. All spectra were recorded in duplicate and averaged.

Turbidity measurements at a protein concentration between 0.2−2.3 wt % were performed at 25 °C on a Shimadzu UV-1800 UV−vis spectrophotometer (Shimadzu Corporation, Japan) equipped with a CPS-temperature controller. Changes in turbidity were measured by following the absorbance at 500 nm in time. Triplicate measurements were carried out on each sample and averaged.

Preparation of Protein Gels. Gelation of unmodified and thiolated structural building blocks prepared from WPI was carried out by acid-induced cold gelation using glucono-δ-lactone (GDL). The concentrations of GDL added to the samples to induce gelation were determined using the formula (% GDL (w/w) = 0.0065 × C protein (g/L) + 0.045).[31] GDL was added to WPI solutions with a protein concentration ranging from ∼0.2 to 2.5 wt % to induce gelation at 25 °C. The pH of all samples was determined after 24 h of incubation at 25 °C in identical samples placed in a water bath at 25 °C.

Rheological Characterization of Protein Gels. A stress-controlled rheometer (ARG2, TA Instruments, Leatherhead, UK) with a concentric cylinder geometry (C14: cup diameter, 15.4 mm; bob diameter, 14.04 mm;) was used to determine storage modulus (G') as a function of time (frequency, 1 Hz; temperature, 25 °C; strain, 0.05, 24 h). After 24 h, oscillation amplitude was performed (frequency, 1 Hz; temperature, 25 °C; strain, 0.01−100). Duplicate measurements were performed on various batches of freshly thiolated aggregates to prevent the possibility of autoxidation of introduced thiol groups occurring during prolonged storage, which could mask the impact of thiolation on gelation efficiency. Measurements were performed within the linear region which was between a strain % of 0.001 and 10.

RESULTS AND DISCUSSION

Thiolation of WPI based fibrillar and spherical aggregates to study its effect on the gelation behavior of WPI structural building blocks yielded blocked (SX) and reactive (SH) WPI variants. These variants were used to describe the impact of thiolation on gel formation.

Chemical Characterization of WPI Fibrils and Spherical Aggregates upon Thiolation. The number of free −SH groups in unmodified and thiolated WPI fibrillar and spherical aggregates was determined using Ellman's reagent, whereas chemical reactivity of introduced thiol groups was determined using sulfhydryl−disulfide exchange index (SEI) (Table 2).

Table 2. Chemical Characterization of WPI Samples Modified with a Ratio of 1:8 S-AMSA/amines − Number of Thiol Groups As Obtained by Ellman's Reagent, Degree of Substitution (DS) % as Obtained by OPA assay, and Thiol Reactivity (%) as Obtained by SEI[a]

	number of thiol groups (mM/mM of protein ± stdev)	DS (%)	reactivity (SEI) (%)
native	0.20 ± 0.03	0	0 ± 0.0
F	0.60 ± 0.07	n.a.	8 ± 0.30
A	0.20 ± 0.02	n.a.	9 ± 0.13
FSX	1.76 ± 0.02	65 ± 3.3	54 ± 0.58
FSH	3.10 ± 0.01	66 ± 2.2	96 ± 1.49
ASX	1.57 ± 0.01	57 ± 0.1	16 ± 0.91
ASH	3.51 ± 0.96	62 ± 1.1	31 ± 0.25

[a]DS = % modified amines of the total number of amines available derived from OPA assay; n.a =not applicable. The number of measurements (n) = 2.

Native WPI had less exposed thiol groups than unmodified fibrils (F). Thiol groups in native β-lactoglobulin, the major fraction of WPI, are reported to be buried between the β-barrel and the C-terminal major α-helix[32,33] and may not be available for reaction with Ellman's reagent. Formation of fibrils from peptides following protein hydrolysis at pH 2 resulted in the increased exposure of thiol groups, suggesting first that conformation of proteins in the fibrillar aggregates may be different from that of the native protein, and second, that exposed thiol groups may function to stabilize interfibrillar interactions upon gel formation. The potential of this concept was further explored by introducing multiple thiol groups onto the fibrillar and spherical aggregates by means of thiolation. Generally, the thiolation procedure led to the introduction of thiol groups to modify approximately 60% of the primary amines available in both WPI fibrillar and spherical aggregates. The number of thiol groups detected by Ellman's reagent increased upon conversion of protected acetyl groups (SX) to SH groups in both fibrillar and spherical aggregates (Table 2).

Upon thiolation, FSX and FSH showed increased thiol reactivity compared to F, whereas thiol reactivity of ASX and ASH was limited (Table 2). FSX showed thiol reactivity of about 50%. The reactivity of FSX, in which thiol groups are blocked, could be due to auto oxidation occurring in the acetyl groups either by reaction with oxygen or reaction of two thiol groups resulting in SH or S−S group.[13] Nevertheless, FSH variants showed higher reactivity compared to F and FSX variants. SEI results of spherical WPI aggregates show that

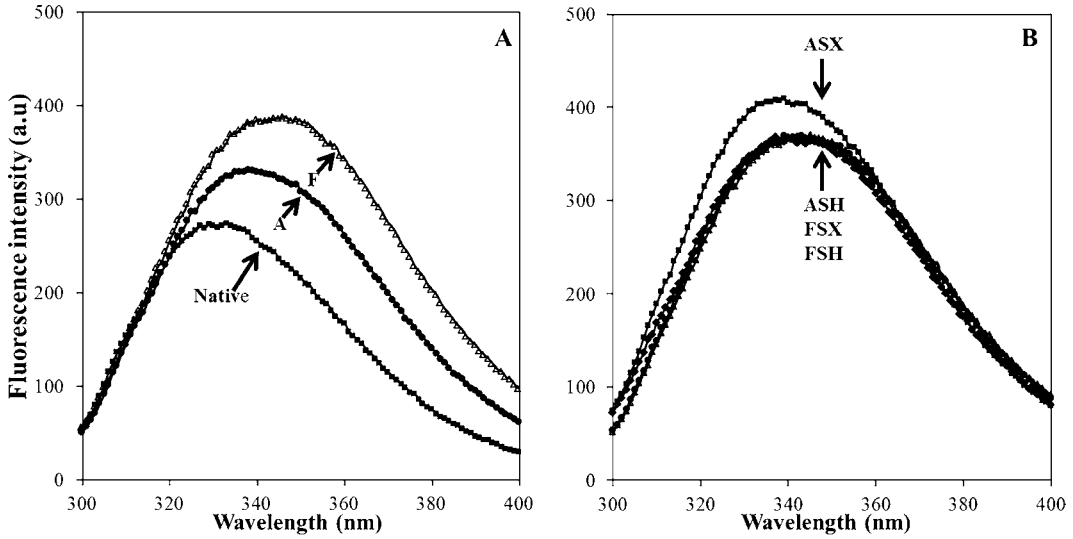

Figure 2. Structural properties of WPI are affected by assembly. Analysis of tertiary structure probed by intrinsic fluorescence for (A) native WPI (native), unmodified WPI fibrils (F), and unmodified spherical aggregates (A); (B) blocked variant of modified fibrils (FSX), deblocked variant of modified fibrils (FSH), blocked variant of modified spherical aggregates (ASX), and deblocked variant of modified spherical aggregates (ASH) variants of WPI.

although thiol groups were successfully introduced in the variants thiolated spherical aggregates appear to have less reactivity than WPI fibrillar aggregates. Attachment of reactive thiol groups to WPI fibrils potentially results in cross-linking reactivity between thiol groups, resulting in disulfide bond formation. Thus, covalent cross-linking could have occurred between and within WPI fibrillar aggregates. These results show that (i) heating of native WPI to form fibrils (from peptides) or to form spherical aggregates increased the exposure of indigenous sulfhydryl groups, and that (ii) deblocking of the SX variant was successful and resulted in increased thiol reactivity.

Structural Analysis of Fibrillar and Spherical Aggregates. The changes in protein folding as a result of aggregate assembly of WPI structural building blocks were probed using intrinsic tryptophan fluorescence (Figure 2). Native WPI exhibited a fluorescence emission maximum at 330 nm which is characteristic for a tertiary folded protein in which the tryptophans are buried in the interior of the molecule as reported for β-lactoglobulin.[34] Conversion of native WPI to fibrillar or spherical aggregates induced a red shift of the fluorescence maximum[35] due to conformational changes that occur as a result of aggregate assembly. The red shift in fluorescence maximum was more pronounced for fibrillar than for spherical aggregates. This observation illustrates that both fibrillar and spherical aggregate formation coincide with conformational change of the tryptophan residues resulting in more solvent exposure of the tryptophans.[35] Unmodified and thiolated WPI fibrillar and spherical aggregates showed higher fluorescence intensity than native WPI. Upon thiolation no further changes were observed in tertiary structure for FSX, FSH, and ASH variants of WPI fibrillar and spherical aggregates compared to unmodified variants (F and A). ASX variant showed the highest fluorescence intensity compared to FSX, FSH, and ASH variants. Variation in fluorescence intensity may be the result of different energy transfer efficiency between tryptophan and tyrosine as a result of structural rearrangement upon fibrillar or spherical aggregate formation as reported for ovalbumin.[23]

A Th T assay was carried out on unmodified and thiolated WPI fibrillar and spherical aggregates to confirm the presence of β-sheet aggregates in protein solutions. Upon association into either fibrillar or spherical aggregates, Th T fluorescence intensity increased compared to the native variant, particularly for WPI fibrillar aggregates (Figure 3). Modification of fibrils resulted in a decrease in the Th T intensity of FSX variant, suggesting a partial disruption of β-sheet fibrillar structure upon thiolation. The partial disruption of β-sheet fibrillar structure could be related to pH changes occurring during the thiolation process. Partial disruption of β-sheet structure related to pH changes has been reported for fibrils made from insulin.[36] A slight increase in Th T intensity was observed in ASX, whereas a reduction in fluorescence intensity was observed in FSH and ASH. The reduction in relative fluorescence intensity in FSH and ASH variants may be due to reduced efficiency of Th T to bind to the protein as a result of inaccessibility of Th T binding grooves.[37]

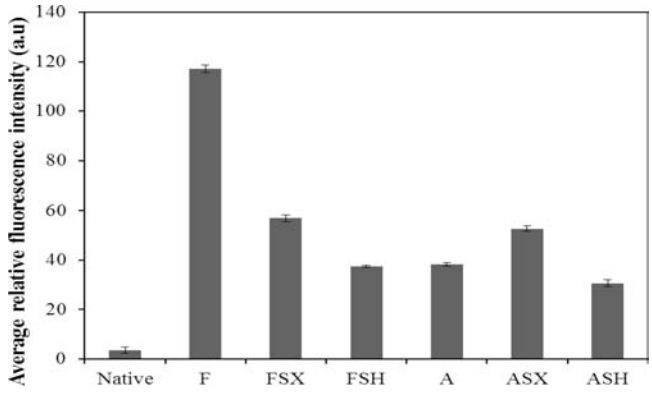

Figure 3. Formation of β-sheet aggregates as detected by Th T is increased upon assembly of WPI. Average relative fluorescence intensities probed by Th T fluorescence at 485 nm wavelength for unmodified (A), thiolated blocked (ASX), and deblocked (ASH) variants of spherical WPI aggregates and unmodified (F), thiolated blocked (FSX), and deblocked (FSH) variants of WPI fibrils.

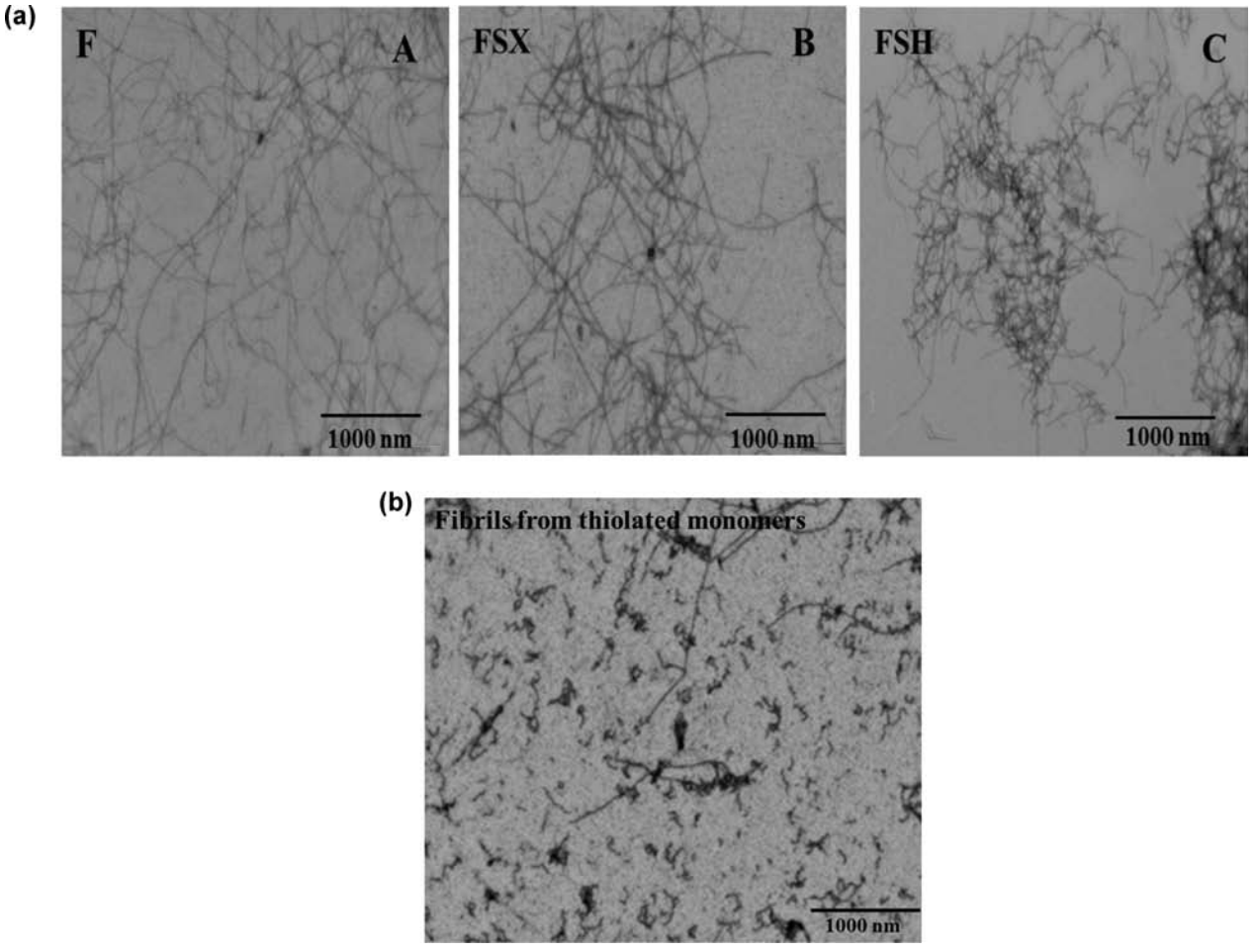

Figure 4. Morphology of fibrillar aggregates. TEM micrographs for (a) unmodified fibrils (F), thiolated fibrils after fibril formation blocked variant (FSX), and deblocked variant (FSH); (b) monomeric WPI thiolated before fibril formation was carried out; at pH ∼ 7. Scale bars represent 1000 nm.

Effect of Thiolation on the Morphology of WPI Aggregates. Fibril morphology was probed using TEM. Fibrils from unmodified WPI (F) show long linear fibrillar aggregates (Figure 4a (A)) that resemble the general kind of fibrils reported before.[1] The ability to form fibrils from WPI that had been thiolated in its "monomeric" state was first assessed (Figure 4b). The TEM micrographs show a mixture of fibrils and small aggregated clusters, suggesting a reduced propensity for fibril formation. Hence, the prospects of thiolation of preformed fibrils were evaluated. The conversion of unmodified fibrils (F) to FSX and FSH resulted in some changes at the fibrillar scale (Figure 4a panels B and C). An apparent increase in clustering and tightening of the adjacent fibrils occurred in FSH as deduced from TEM images, suggesting that enhancement of physical interactions occur following unblocking of the introduced thiol groups.

The use of TEM to study the effect of thiolation on microstructure of spherical aggregates did not show differences between unmodified and thiolated spherical aggregates. Cryo-TEM images for spherical WPI aggregates have been reported before.[13] The expected high level of clustering for spherical aggregates resulted in the use of dynamic light scattering instead of TEM to evaluate the aggregate size upon thiolation. The average hydrodynamic diameters of the largest intensity peak were 38 nm for A (with a % polydispersity index of 62), 68 nm for ASX (with a % polydispersity index of 62), and 106 nm for ASH (with a % polydispersity index of 64) (Figure 5). Thiolation increased the spherical aggregate size and their clustering. These observations suggest that physical interactions occur upon thiolation that result in the clustering of adjacent aggregates.

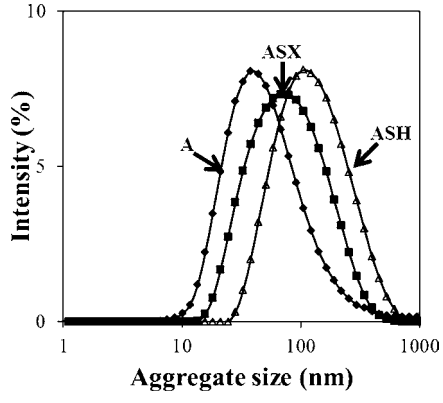

Figure 5. The hydrodynamic radius of aggregates is increased upon thiolation. Aggregate size of unmodified spherical WPI aggregates (A), blocked (ASX), and deblocked variants (ASH) of thiolated spherical aggregates determined by dynamic light scattering.

Effect of Thiolation on Gelation of WPI Fibrils and Spherical Aggregates. Evaluation of whether thiolated preformed WPI fibrils are capable of forming gels at lower protein concentrations than unmodified preformed fibrils (F) was carried out. The spatial clustering of aggregates and gelation of a range of protein concentrations of F were investigated using turbidimetry and small deformation rheology. The turbidity and storage modulus (G') were followed as a function of time, after the addition of GDL to induce the gelation process. During acidification, an initial increase in turbidity was observed for all concentrations indicating an increase in aggregate size (Figure 6). As the pH values

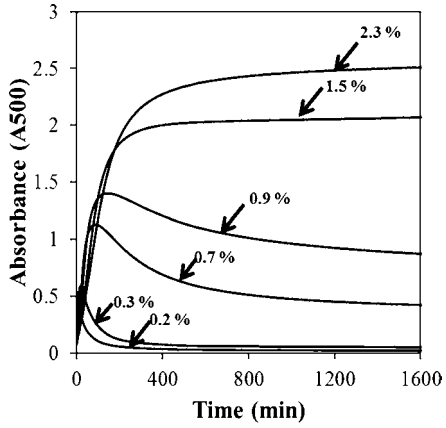

Figure 6. The formation of turbid networks is increased with increasing protein concentration. Changes in turbidity over time at A500 nm for various protein concentrations of unmodified WPI fibrils (F) from 0.2 wt %, 0.3 wt %, 0.7 wt %, 0.9 wt %, 1.5 wt %, and 2.3 wt %.

decreased to below the IEP of WPI, turbidity of 0.2, 0.3, 0.7, and 0.9 wt % of F decreased. The decrease in turbidity could be related to solubilization of the fibrils that are clustered at pH around the IEP. A similar observation of disassociation at pH < IEP has been reported elsewhere.[38] The final turbidity of unmodified WPI fibrils increased with increasing protein concentrations. At low protein concentration (0.2 and 0.3 wt %), the solutions remained transparent. At higher protein concentrations (0.7 and 0.9 wt %), turbidity increased. Persistent turbid gels were only formed at concentrations of 1.5 and 2.3 wt % as the turbidity increased steadily with ongoing gelation and leveled off after 20 h (Figure 6).

The development of G' was followed in time as a measure of gelation for different protein concentrations for both fibrillar and spherical aggregates. Gelation was assumed to have occurred when tan δ of the solution was less than 1 ($G' > G''$).[39] Figure 7 shows the G' values attained after 24 h of gelation at 25 °C as a function of protein concentration for WPI fibrillar (Figure 7A) and spherical aggregates (Figure 7B). With increasing protein concentrations, G' values obtained after 24 h gelation also increased. The increase in G' as a function of concentration was higher for FSX than for F. For example, at a concentration of about 0.9 wt % the G' obtained for FSX was 600 Pa, whereas that of F variant was 265 Pa (Table 3 and Figure 7). Deblocking of the thiol groups to FSH did not result in a further increase in G' (Table 3). Similar trends were observed for unmodified (A) and modified (ASX) variants of spherical aggregates (Figure 7B), although the resultant gels were weaker for spherical aggregates compared to fibrillar

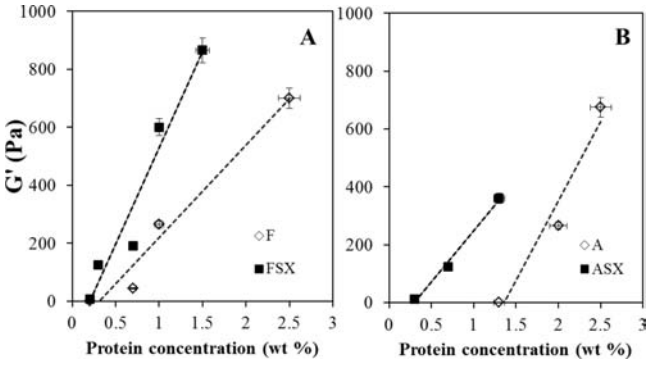

Figure 7. Thiolation of preformed fibrillar and spherical aggregates affects gel properties (I). Changes in final G' (Pa) values obtained after 24 h of GDL induced gelation as a function of protein concentration for (A) unmodified WPI fibrils (F) and blocked variant of thiolated WPI fibrils (FSX); (B) unmodified spherical aggregates (A) and blocked variant of thiolated spherical aggregates (ASX). The error bars represents standard deviation from the mean of duplicate measurements performed on different batches of samples thiolated in a similar way.

Table 3. Final G' (Pa) of WPI Variants Following Acidification Using GDL and Onset Gelation Time (min) at 0.9 wt % Protein Concentration[a]

WPI variant	final G' (Pa, after 1600 min)	onset gelation (min)
F	265 ± 2	120
FSX	600 ± 4	285
FSH	664 ± 3	116
A	1 ± 0.12	25
ASX	360 ± 1	60
ASH	45 ± 3	260

[a]F is fibrillar aggregates, and A is spherical aggregates.

aggregates at similar protein concentrations. Thiolation appeared to effectively reduce the critical gel concentration compared to unmodified fibrillar and spherical aggregates, which could be attributed to increased potential of chemical cross-linking between fibrillar and spherical aggregates upon thiolation.

It has been reported that the conversion of peptides to fibrils is not as efficient as the conversion of monomeric proteins to spherical aggregates, as over 95% of monomeric protein is converted into spherical aggregates following the heating of proteins[14] compared to about 50% conversion of peptides to fibrils.[9] To ensure that the observed effects of thiolation on gelation of fibrils had no interference from nonconverted peptides, WPI fibrils were "purified" as previously described.[40] The results show that the presence or absence of non-aggregated proteins/peptides in the samples does not interfere with either thiolation or gelation of WPI fibrillar aggregates (see Supporting Information).

Comparing fibrils to unmodified and thiolated spherical aggregates, it can be observed that thiolation of spherical aggregates also reduces gelation concentration and increases final gel strength (Figures 7B and 8), even though thiol reactivity was not significantly improved (Table 2). At similar protein concentration, fibrillar aggregates formed stronger networks than spherical aggregates (Table 3). For example, at a concentration of about 0.9 wt % no gels were formed for A, whereas ASX formed gels with a G' of 360 Pa.

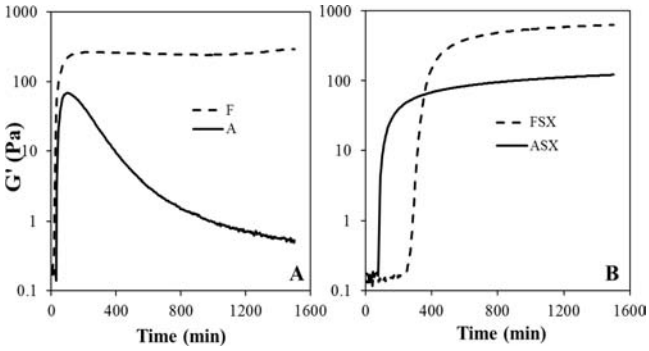

Figure 8. Thiolation of preformed fibrillar and spherical aggregates affects gel properties (II). Changes in G' (Pa) over time for GDL-induced gelation of WPI at a protein concentration of 0.9 wt % for (A) unmodified spherical aggregates (A) and fibrils (F); (B) blocked variants of spherical (ASX) and fibrillar (FSX) aggregates.

Fibrils have been shown to form gels at low protein concentration.[15] It has been reported that fibrils are capable of forming gels with an order of magnitude lower protein concentrations than conventional cold- or heat-induced gelation of aggregates upon the introduction of an extra attractive interaction.[15] Such additional attractive interactions were introduced in this article by means of thiolation. Fibrillar forms of WPI, regardless of whether they were thiolated or not, always formed gels with higher G' values than gels formed from spherical aggregates. Gelation of ASX and ASH resulted in formation of weaker gels than FSX and FSH, at a similar protein concentration. The final gel strength for FSX and FSH was higher than ASX and ASH. FSH had comparable gel firmness as FSX, whereas ASH gels were weaker than ASX (Table 3). Formation of weaker gels by ASX and ASH can be attributed to the differences in the reactivity of thiol groups as lower chemical reactivity was observed in ASX/ASH as opposed to FSX/FSH (Table 2). Additionally, differences in the curvature of spherical aggregates in comparison to linear fibrillar aggregates have been shown to lead to lower gel stiffness in ovalbumin.[23]

Gelation kinetics between ASX, FSX, ASH, and FSH were different (Table 3). Observed differences in gelation kinetics and gel firmness for ASX and ASH could be related to deblocking of the introduced sulfhydryl groups, which could enhance the possibility of reaction of adjacent −SH groups resulting in disulfide bond formation. The permanent nature of disulfide bonds does not provide rotational freedom in the gel network which can result in rearrangement of the hydrophobic interactions to form a structure that can optimize gel strength due to the high disulfide bond energy.[23]

In conclusion, it can be stated that SH/SS groups play an important role in determining the strength of GDL-induced protein gels. The influence of SH/SS on gel formation was more pronounced in networks made from fibrillar than from spherical aggregates. This study has shown that a combination of thiolation with GDL-induced gelation decreases gelation concentration and increases final gel strength of WPI fibrillar and spherical aggregates. The results show that the capacity of WPI as a structuring agent can be increased by thiolation of preformed fibrillar and spherical aggregates. Thus, proteins as food ingredients could be employed more broadly to attain new food structures by increasing their chemical cross-linking potency by means of thiolation. However, thiolated protein aggregates have to be tested on toxicological and organoleptic properties in order to be regarded as safe to use in food applications.

■ ASSOCIATED CONTENT

Supporting Information

Figure 1: Formation of β-sheet aggregates as detected by Th T is increased upon assembly of WPI into fibrils. Figure 2: Thiolation of preformed fibrils affects gel properties. Table S1: Overview of unmodified purified WPI fibrils and thiolated (SX-blocked and SHdeblocked variants) WPI fibrils essentially free from nonaggregated proteins/peptides. Table S2: Physico-chemical characterization of WPI samples modified with a ratio of 1:8 S-AMSA/amines − degree of substitution (DS) % and thiol reactivity (%). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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