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Towards the understanding of molecular mechanisms in the early stages of heat-induced aggregation of β-lactoglobulin AB

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Abstract

Heat-induced aggregation of bovine β-lactoglobulin AB (10 mg/ml) was studied at 68.5 °C at two different pH values (6.7, 4.9) using gel electrophoresis techniques and matrix-assisted laser desorption ionization mass spectrometry (MALDI–TOF MS). Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) analysis under non-reducing and reducing conditions showed that in the early stages of the aggregation of β-lactoglobulin disulfide linked aggregates were formed on heating at pH 6.7, but not at pH 4.9. We related this result to the pH-dependent activity of the free thiol group at C121. Mass spectrometric analyses were conducted in two steps. The first involved the analysis of intact non-native monomers and dimers following their ultrasonic passive elution into a suitable solvent mixture in order to confirm the identity of the different gel bands. The second step comprises the analysis of in-gel digests for the determination of disulfide patterns in non-native monomers, covalent dimers and trimers. The results of in-gel digestions analyzed by mass spectrometry suggest that non-native dimers could result from the formation of inter-molecular disulfide bonds C121–C66, C160–C160, or C121–C160. Moreover, two inter-molecular bonds C121–C66 and C160–C160 between two and the same monomer units have been detected, which may play an important role in limiting the process of covalent β-lactoglobulin network formation. The combination of SDS–PAGE and MALDI–TOF MS enables us to understand the mechanism of β-lactoglobulin aggregation at the macromolecular level.

Keywords: Heat-treatment; Aggregate formation; Disulfide bonds; β-Lactoglobulin

1. Introduction

Whey proteins are proteins that remain soluble after rennet or acid precipitation of caseins from milk. The major whey proteins are β-lactoglobulin (β-Lg), α-lactalbumin, and bovine serum albumin. Whey proteins are used as functional ingredients in foods, and the effect of heat treatment on their functional properties is essential in a variety of applications of these proteins [1]. Depending on the experimental conditions, such as the time of heating, temperature, pH, and the presence of other compounds, these proteins can remain in solution or form aggregates, gels, or precipitates. Consequently the effect of heat on whey proteins can have a significant impact on dairy processing, e.g. in cheesemaking.

β-Lg is the major protein in whey (50% of the total whey protein in bovine milk) and dominates the overall aggregation and gelation behaviour of whey

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protein preparations. β-Lg is a globular protein belonging to the lipocalin family. Monomeric bovine β-Lg consists of a single peptide chain of 162 amino acids and has a molecular mass of about 18.3 kDa. It occurs in two natural variants β-Lg A (18 367 Da) and β-Lg B (18 281 Da), each containing two disulfide bridges (C66–C160 and C106–C119) and one free cysteine (C121) [2,3]. Structure determination of β-Lg has been carried out by X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy at neutral and low pH, respectively [4–10], revealing the presence of a very stable β barrel, consisting of nine antiparallel β sheets and a C-terminal α-helix. At room temperature and physiological conditions, β-Lg exists mainly as a non-covalently linked dimer. Upon heating to approximately 65–70 °C, the dimers dissociate into monomers, which partially unfold, resulting in a molten globule state. These critical changes in the conformation of β-Lg lead to the exposure of the previously buried inner hydrophobic groups of the molecule and the free thiol group at C121. The exposed C121 in the denatured monomer can induce thiol/disulfide exchange reactions, leading to the formation of disulfide-linked aggregates and polymers. The occurrence of thiol/disulfide exchange reactions in heated β-Lg and their involvement in the heat-induced aggregation and gelation of β-Lg have been demonstrated [11–16]. Roefs and de Kruif [17] proposed a thiol-catalyzed aggregation mechanism for β-Lg at 65 °C, neutral pH and low ionic strength, analogous to free-radical-induced polymerization. In addition to this covalent inter-molecular coupling, non-covalent interactions (ionic, van der Waals, hydrophobic) may also be involved. The extent of their relative contribution to the overall aggregation and gelation process is still unclear and depends on experimental conditions such as pH and salt concentration. The kinetics of β-Lg thermal denaturation, aggregation and the effect of heat treatment on native β-Lg have been extensively studied using a range of different physical techniques including light scattering [17–20]; circular dichroism spectroscopy [21–26], intrinsic protein fluorescence [23], hydrophobic probes [23], ellipsometry [27], calorimetry [28–30], and NMR [24,31]. However, the molecular mechanism of heat-induced aggregation of β-Lg has yet to be fully determined.

In literature, studies towards conformational changes and inter-molecular crosslinking, of β-Lg often include partial enzymatic hydrolysis followed by identification of the disulfide bond positions in the peptides formed. Table 1 presents a list of several authors who have identified peptides by enzymatic hydrolysis of β-Lg. Some of these peptides contained the disulfide bond C66–C160 originally present in the parent protein [32,33]. No disulfide-linked peptides have been identified in β-Lg hydrolysates formed by enzymes other than trypsin and plasmin, such as bromelain, papain, pepsin, or endoproteinase Arg-C [33]. Turgeon et al. [32] suggested the formation of new disulfide bonds during tryptic hydrolysis of β-Lg at pH 8 due to the increased reactivity of the free thiol group at C121. Morgan et al. [34] discovered that heated β-Lg (at 60 °C and pH 7) contained the non-native C160–C160 and C160–(C121 and/or C119 and/or C106) disulfide bonds after tryptic digestion at pH 8.5. Maynard et al. [35] produced evidence for crosslinking between C121 and C160 after tryptic digestion at pH 7.7 and different temperatures. Caessen et al. [36] observed newly formed inter-molecular disulfide bonds (C66–(C121 and/or C119 and/or C106); C160–(C121 and/or C119 and/or C106); C66–C66) during hydrolysis of β-Lg by plasmin at pH 8. Although conditions used in these studies were all different,

Table 1
Selected studies involving β-Lg hydrolysis in which intra- or inter-molecular disulfide bonds were identified

Reference	Preheating conditions	Digestion conditions	Disulfide bridge positions identified
Turgeon et al. [32]	/	Trypsin, 37 °C, pH 8	C66–C160
Otte et al. [33]	/	Bromelain, Papain, Trypsin, Arg-C . . . , pH 8	C66–C160 (Trypsin)
Morgan et al. [34]	60 °C, pH 7	Trypsin, 37 °C, pH 8.5	C160–C160, C160–C121/C119/C106
Maynard et al. [35]	/	Trypsin, 37 °C, pH 7.7	C121–C160
Caessens et al. [36]	/	Plasmin, 37 °C, pH 8	C66–C121/C106/C119

they provide evidence for the occurrence of thiol/disulfide exchange reactions under slightly basic conditions. From these studies, it is, however, unclear what inter-molecular disulfide bridges are being formed when β-Lg solutions are heated at various defined pH conditions.

The aim of this study was to investigate the early stages of the heat-induced aggregation of β-Lg and to give a better understanding about the thiol/disulfide exchange mechanism at conditions of concentration, pH and heating, relevant in food processing. The characterization of β-Lg aggregates is accomplished using SDS–PAGE followed by mass spectrometry. In-gel digestion of the separated covalent oligomers by trypsin under appropriate conditions followed by mass spectrometric analysis of the peptides formed are used for identifying the position of inter- and intra-molecular covalent bonds.

2. Experimental

2.1. Materials

In all experiments, a purified bovine β-Lg sample, containing the genetic variants A and B, was used, purchased from Sigma (St Louis, MO, USA). The materials used in the present investigation were:

Trypsin sequencing grade, Tris (Roche Diagnostics, Mannheim, Germany), acrylamide, *N*,*N*′-methylenebis-(acrylamide), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), ammonium persulfate, bromophenol blue, molecular mass calibration kit (all from Bio-Rad Laboratories, Hercules, CA, USA), sodium dodecyl sulfate (SDS), dithiothreitol (DTT) (Sigma, St Louis, MO, USA), sinapinic acid (Aldrich, Dorset, UK), glycine (Merck Eurolab, Poole, Dorset, UK), and Coomassie Brilliant Blue R-250 (Merck Eurolab, Poole, Dorset, UK). All other reagents and solvents were of the highest available grade. The water was purified by Milli-Q (Millipore, Watford, Herts, UK) with resistivity >18 MΩ.

2.2. Heat treatment

The β-Lg solutions used for the experiments contained 10 mg/ml of protein in 25 m*M* ammonium acetate and were adjusted to pH 4.9 or 6.7. Aliquots (0.2 ml) of β-Lg in glass vials were incubated at 68.5 °C for different times in a thermostatically controlled water bath. The vials were closed tightly to prevent evaporation during the incubation. After the heating period, each aliquot was immediately quenched in ice bath and mixed with equal volumes of SDS–PAGE sample buffer. Then, the mixture was stored at 4 °C for less than 10 h until electrophoresis.

2.3. Electrophoresis

The β-Lg solutions were analyzed by SDS–PAGE using a Hoefer SE600 system (Amersham Pharmacia Biotech, Little Chalfont, UK). In order to create reducing conditions DTT was added (Protein/DTT, 1:500 molar ratio) and the mixtures were incubated for about 1 h at room temperature before electrophoresis.

2.3.1. Preparation of SDS–PAGE gels

The resolving gel was made from a mixture of 33.3 ml of a 30% stock solution of a 35.5:1 mixture of acrylamide and *N*,*N*′-methylenebis-(acrylamide), 20 ml of resolving gel buffer [1.5 *M* Tris–Cl adjusted to a pH of 8.8], 0.8 ml of 10% (w/v) SDS solution and 25.5 ml of water. Just prior to the gels being poured, 400 μl of 10% (w/v) ammonium persulfate solution and 27 μl of TEMED were added to the mixture. The gel-setting apparatus was assembled using 1.5-mm spacers, and the resolving gel solution was put between each pair of glass plates to a level of about 4 cm from the top. About 0.5 ml of water-saturated *n*-butanol was then placed above the resolving gel solution and the apparatus was set aside at room temperature (20–25 °C) for the gels to set, which took ~1 h. The overlying water-saturated *n*-butanol was poured off by tilting the casting stand and the surfaces of the gels were rinsed twice with water. The water was then drained off with the aid of a paper wick.

The stacking gel was made from a mixture of 2.66 ml of the 30% stock solution of acrylamide and bis (acrylamide), 5 ml of stacking gel buffer (0.5 *M* Tris adjusted to pH 6.8), 0.2 ml of 10% (w/w) SDS solution and 12 ml of water. Then, 100 μl 10% (w/v) ammonium persulfate solution and 10 μl of TEMED were added. This mixture was pipetted into the gap between the glass plates, and the slot former

was inserted, taking care that no air bubbles were entrained. Once the gel had set, the gel formers and gels were put in a 4 °C cold room overnight. The electrode buffer solution was prepared as 0.025 *M* Tris/0.192 *M* glycine and 0.1% (w/v) SDS solution and adjusted to pH 8.3. The SDS–PAGE sample buffer comprised 0.125% *M* Tris (adjusted to pH 6.8), 0.02% bromophenol blue, 4% SDS and 20% glycerol.

2.3.2. Loading, running, staining, and scanning the gels

A 7-μl aliquot of each sample (already mixed with SDS sample buffer) was loaded in each well. The electrophoretic conditions were 20 mA for 15 min, followed by 80 mA for 4 h, running two gels at the same time. After electrophoresis, gels were stained with 0.1% Coomassie Brilliant Blue R250 according to the instructions of the manufacturer. Gels were scanned and treated by Bio-Rad Quantity One (Bio-Rad Laboratories, Hercules, USA).

2.4. Elution of intact proteins from polyacrylamide gel

The protein–gel bands were destained with water/methanol/acetic acid (50:40:10, v/v) at room temperature, frozen at −80 °C, crushed and then incubated in 40 μl of an extraction solvent, consisting of formic acid/acetonitrile (40:60, v/v). The incubation was performed in an ultrasonic bath for 50 min at room temperature. Part of the resulting solution was used for mass spectrometric analysis.

2.5. Tryptic digestion

The stock solution of trypsin (1 mg/ml, pH<3) was prepared and stored as proposed by the manufacturer. The protein bands of interest from stained polyacrylamide gel were excised. Each gel piece was cut into small particles (~1 mm^2) using a scalpel, and placed into a 0.5-ml Eppendorf tube. The gel particles were immersed in 50 m*M* ammonium acetate/50% acetonitrile solution and sonicated for 10 min. A gel loading pipette tip was used to remove the solution. This wash/dehydration step was repeated up to three times. Then 1 μl of trypsin (1 mg/ml) was added and gel particles were overlaid with a volume (~50 μl) of 50 m*M* ammonium acetate (pH 6.7) to keep them immersed during digestion. The digestion was performed overnight at 37 °C.

After adding 50 μl water, the Eppendorf tube was vortexed for 5 min and then sonicated for 5 min. Using a gel-loading tip, the peptide solution was removed and transferred to another Eppendorf tube. Two additional extractions using 50 μl of water/TFA/acetonitrile (39.9:0.1:60, v/v/v) during 30 min were carried out and the combined peptide extracts were dried in a vacuum centrifuge. The recovered peptides were dissolved in 10–15 μl 0.1% TFA, concentrated and cleaned using C_{18} Zip Tips (Millipore, Bedford, USA).

2.6. Mass spectrometry

All mass spectra were recorded using a Voyager-DE STR MALDI–TOF mass spectrometer (Applied Biosystems, Foster City, CA, USA) operated in delayed extraction mode. Ionization was initiated with a nitrogen laser operating at 337 nm with a repetition rate of 3 Hz. Delayed extraction linear mode with an accelerating voltage of 25 kV was used for measurements of the intact monomers and dimers resulting from the ultrasonic assisted passive elution. Spectra of the tryptic digests were recorded in positive reflectron mode, using an acceleration voltage of 20 kV.

Stock solutions of MALDI-matrices were prepared fresh each day by dissolving 10 mg of sinapinic acid in 1 ml of water/TFA/acetonitrile (39.9:0.1:60, v/v/v). For analysis of intact proteins, the three-layer deposition method was used, where the protein solution was sandwiched between two layers of matrix solution in the following manner: (i) 0.6 μl of matrix solution was applied on the sample deposition plate and allowed to dry; (ii) 1 μl of the sample protein was applied onto the same spot and allowed to dry; (iii) another 0.6 μl of matrix solution was deposited on the spot. For analysis of the tryptic digests, 2 μl of the matrix solution was used to elute peptides from C_{18} ZipTips (Millipore) and deposit them on a flat gold metal plate which was left at room temperature long enough to allow solvent evaporation.

3. Results and discussion

3.1. Formation of disulfide-linked aggregates and its pH dependence

Heated solutions of β-Lg (10 mg/ml) were analyzed by SDS–PAGE under non-reducing and reducing conditions to verify the involvement of disulfide bond formation in the aggregation of β-Lg. The buffer system used for separation under non-reducing conditions contains SDS which causes disruption of non-covalent bonds while aggregates linked through disulfide bonds remain intact.

Results of SDS–PAGE analysis under non-reducing conditions for β-Lg solutions, heated for 0, 0.25, 0.5, 1, 3, 6 h at 68.5 °C at pH 6.7, are given in Fig. 1A. In the unheated sample, a monomer band and a faint dimer band can be observed. With increasing heating time, the decline of monomeric β-Lg is accompanied by an increase in higher aggregates of different sizes. Polymeric aggregates with a molecular mass greater than 300 kDa fail to migrate into the stacking and separating gel. The presence of multiple bands for dimers, trimers and oligomers, respectively, indicates a considerable extent of inter- and intramolecular disulfide bond diversity. It should be noted that the concentration of dimer band gradually increases with heating time up to ~1 h after which it decreases. The trimer band appears at 0.25 h, reaches an optimum in concentration at ~1 h and decreased thereafter. Higher oligomers which are not able to penetrate the separating gel clearly appear at ~1 h (simultaneous with the maximum in the concentrations of dimers and trimers); these oligomers reach a maximum concentration at ~3 h and then decrease.

In Fig. 2, relative concentrations of various mono-, di-, tri-, oligo- and polymeric species of β-Lg formed during heating at 68.5 °C versus heating times are presented to visualize the kinetic aggregation behavior. It appears that polymers begin to form when the concentrations of dimers and trimers reach a maximum. These results suggest that, under the conditions used, critical concentrations of dimers, trimers, and oligomers are required to initiate polymer formation. Apparently dimers, trimers and higher oligomers act as the building blocks for the formation of polymers. This is in agreement with results described by Prabakaran et al. [25], Bauer et al. [37] and Roefs and de Kruif [17].

When separated under reducing conditions (data

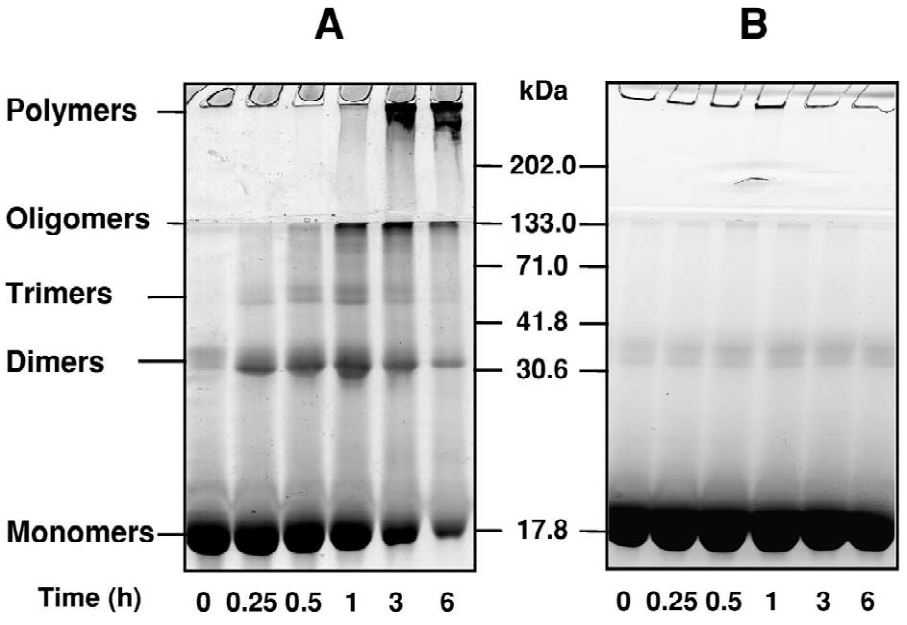

Fig. 1. SDS–PAGE analysis of β-Lg solutions in 25 m*M* ammonium acetate adjusted to pH 6.7 (A) and pH 4.9 (B), and heated for various times at 68.5 °C. The electrophoresis was run under nonreducing conditions.

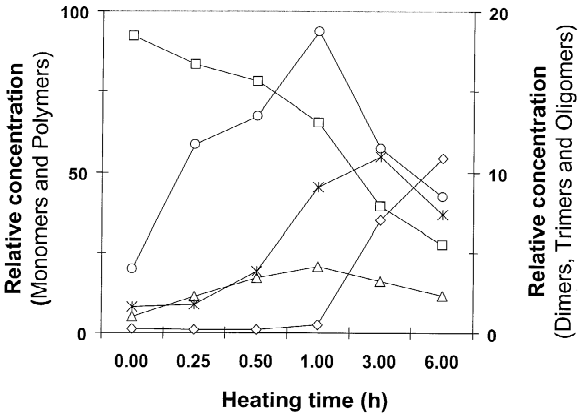

Fig. 2. Relative concentrations of monomeric (□), dimeric (○), trimeric (△), oligomeric (*) and polymeric (◊) β-Lg in ammonium acetate buffer, pH 6.7 as a function of heating time at 68.5 °C. The data were derived from relative intensities of protein bands presented in Fig. 1A.

not shown) all the β-Lg samples appear to contain exclusively β-Lg monomers indicating that disulfide bonds are responsible for the inter-molecular interactions of β-Lg induced by heating at pH 6.7.

One of the most important ways to build structure with milk proteins is acidification (either by means of lactic acid bacterial cultures or addition of acid). Acidification of milk results ultimately in the coagulation or gelation of the casein micelles at pH 4.6. When milk is submitted to heat-treatment (70–90 °C) prior to acidification, a shift in gelation pH to more alkaline values is observed. β-Lg seems to be responsible for this shift by interacting with casein micelles (involving κ-casein) through inter-molecular bonds [38]. It is not known whether β-Lg heated under acidic conditions can form covalently bonded complexes, then further contributing to the gelation process. Therefore, we investigated whether heating under acidic conditions could induce β-Lg aggregation. β-Lg solutions, heated for 0, 0.25, 0.5, 1, 3, 6 h at 68.5 °C and pH 4.9, were analyzed by SDS–PAGE under non-reducing conditions (Fig. 1B). In all samples, heat treatment did not induced any aggregation or polymerization of β-Lg. Heating of β-Lg solutions at pH 4.9 to approximately 65–70 °C leads to the exposure and accessibility of the free thiol group C121 but this thiol group apparently does not initiate any thiol/disulfide exchange. Comparing the results of Fig. 1A (pH 6.7) and Fig. 1B (pH 4.9), it appears clearly that the reactivity of the thiol group depends strongly on pH during heating.

Gel patterns under reducing conditions (data not shown), obtained for β-Lg solutions heated at pH 4.9, are identical to those obtained under non-reducing conditions.

3.2. MALDI–TOF analysis of the intact oligomers

SDS–PAGE is the most commonly used analytical technique for fast separation of peptides, proteins and mixtures of them. However, the accuracy for mass determination is rather limited (to about 1–10% or even worse) as the mobility of the molecules to be separated is governed by their hydrodynamic volume and structure. Accurate mass analysis of gel separated proteins can be greatly improved using MALDI–TOF MS. The bands containing monomers and covalent dimers of β-Lg were excised from the gel and directly extracted by an ultrasonic-assisted passive elution in a solvent mixture composed of formic acid/acetonitrile (40:60, v/v). MALDI–TOF mass spectra of the extracted monomers and dimers, present after 1 h heating at 68.5 °C, pH 6.7 (obtained from the lane corresponding to 1 h in Fig. 1A), are shown in Fig. 3B and 3A, respectively. These spectra exhibit predominantly singly protonated molecular ions representing intact β-Lg monomer (m/z 18 369 Da) and dimer (m/z 36 718 Da) with some adducts due to complexation with Coomassie Blue and/or MALDI matrix (sinapinic acid). β-Lg monomers corresponding to B and A variants are not completely separated. It appeared to be difficult to obtain suitable mass spectra from higher oligomeric bands, probably because of the occurrence of adducts and/or due to low extraction yields.

3.3. MALDI–TOF analysis of in-gel digests

To determine the mechanism of disulfide bond formation during the early stages of heat-induced aggregation of β-Lg, non-native monomers, covalent dimers and trimers were subjected to in-gel digestion and the peptide fragments formed were analyzed by MALDI–TOF MS. As already mentioned, the different SDS–PAGE bands representing the covalent dimers and trimers, respectively, will probably con-

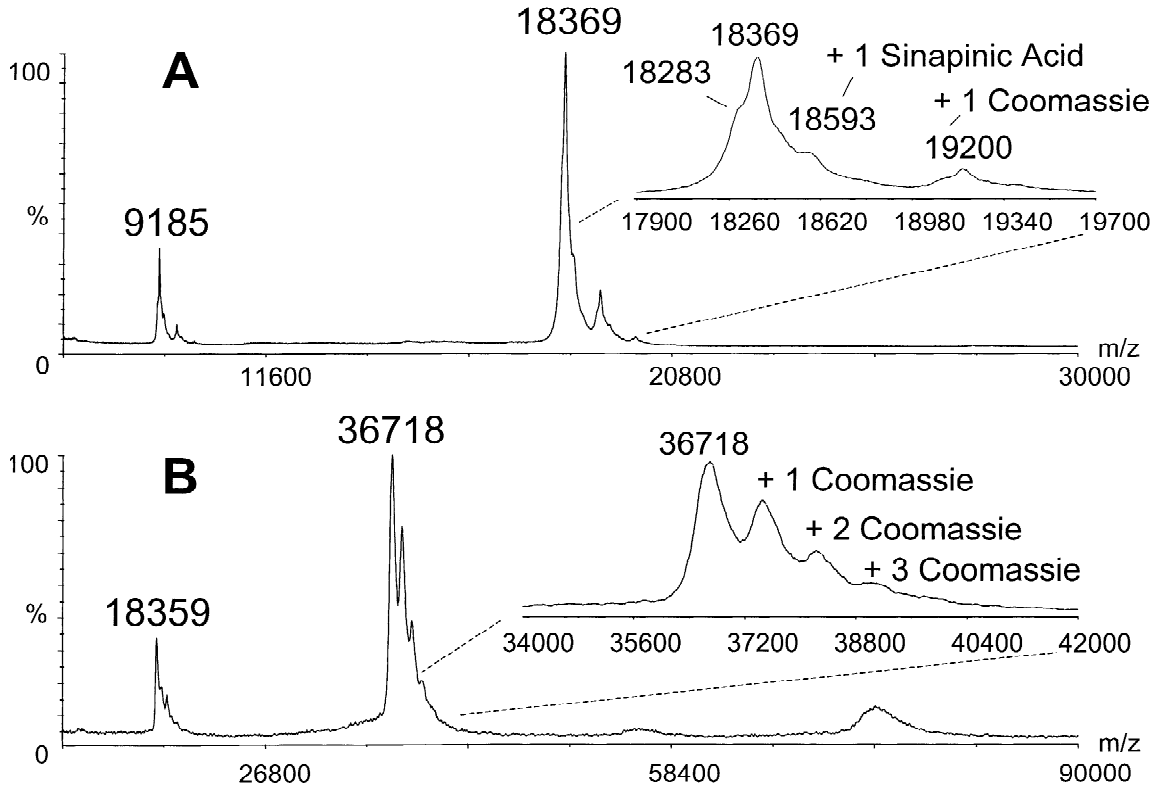

Fig. 3. MALDI–TOF mass spectra of (A) β-Lg monomer and (B) covalent β-Lg dimer extracted from SDS–PAGE gels by ultrasonic assisted passive elution.

tain differently linked oligomers. As it is known already that substantial reshuffling of disulfide bonds can take place during trypsin hydrolysis at pH 8, the in-gel tryptic digestions were carried out at pH 6.7.

Partial MALDI–TOF mass spectra showing relevant peptide fragments obtained by in-gel tryptic digestions from the gel bands containing the monomers, dimers and trimers, present after heating for 0.5 h (corresponding to lane 0.5 h in Fig. 1A) are presented in Fig. 4. The spectrum of the tryptic peptides obtained from the monomer band (Fig. 4C) clearly shows ions at m/z 5143.48 and m/z 5200.46 representing the sequence [41–70]S–S[149–162] from β-Lg B and A variants, respectively. Consequently, these fragments contain the intact, naturally occurring intra-molecular disulfide bond between C66 and C160. The peaks at m/z 2645.19 and m/z 2673.21 were assigned to the sequence [102–124] in the B and A variants, respectively. These peptides contain the three residues C121, C106 and C119. It can be assumed from the exact mass measurement that the intra-molecular disulfide bond between C106 and C119 is still intact and that C121 is present in its free thiol form. If all cysteines were reduced, the theoretical mass of this peptide would be 2647.18 and 2675.21 Da for the B and A variants, respectively.

In principle, the free thiol at C121 could have been involved in thiol/disulfide rearrangements, e.g. during extraction from the gel and proteolysis but there is no evidence that such artefacts have occurred at a significant level under the experimental conditions used. The only indication for a slight degree of thiol/disulfide reshuffling are the minor peaks at m/z 4300.73 and m/z 4328.75. These peaks represent the sequence [102–124]S–S[149–162] in the B and A variants, respectively, thus indicating the formation of a disulfide bond between C121 and

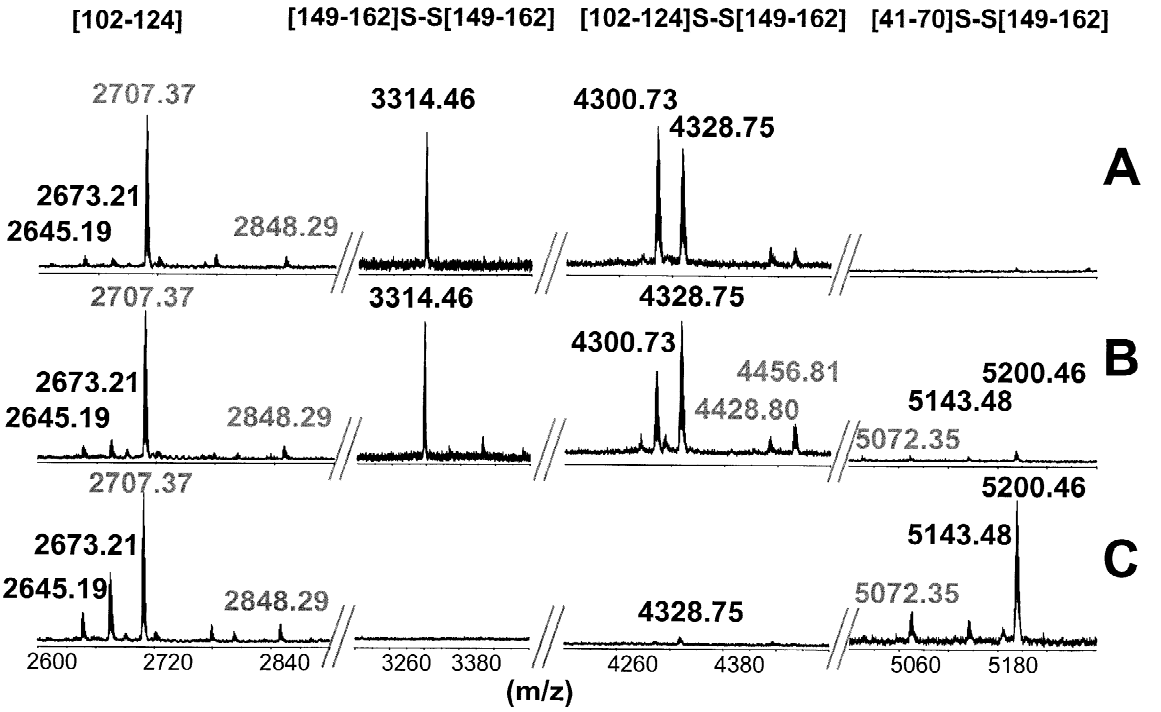

Fig. 4. Partial reflectron MALDI–TOF mass spectra of in-gel tryptic digests of (A) covalent β-Lg trimers, (B) covalent β-Lg dimers and (C) monomers formed by heating at pH 6.7.

C160 (see also the spectra of dimers and trimers). However, their intensity is negligible.

The identification of the peptide components present in the covalent dimer hydrolysate is given in Table 2. The MALDI–TOF spectrum contains nearly 40 peptide signals. Many of them represent peptides linked by intra- or inter-molecular disulfide bridges.

Partial MALDI–TOF mass spectra showing relevant peptide fragments of the dimer and trimer hydrolysates are presented in Fig. 4B and 4A, respectively. Some peptides have been identified containing newly formed intra- and inter-molecular disulfide bonds. The peaks at m/z 4300.73 and m/z 4328.75 represent peptides [102–124]S–S[149–162] containing an inter-molecular disulfide bond between C121 and C160 (Table 2). These peptides are almost absent in the spectrum of the monomer hydrolysate (Fig. 4C). As previously mentioned, the formation of an inter-molecular disulfide bond between C121 and C160 under the experimental conditions used was expected due to the high reactivity of these cysteine residues [39]. The reduced intensity of the peaks at m/z 2645.19 and 2673.21 (fragment [102–124]) in Fig. 4A and 4B, compared to the monomer spectrum (Fig. 4C) should not be explained by the disruption of the disulfide bond between C106 and C119 but rather by the incorporation of this sequence in the inter-molecular fragment [102–124]S–S[149–162] (m/z 4300.73; m/z 4328.75). The peak at m/z 3314.46, present in the mass spectra of dimer and trimer hydrolysates, is assigned to the sequence [149–162]S–S[149–162] (the same for variants A and B) and points to the formation of a disulfide bond between C160 and C160. For the covalent dimer, it is surprising to find an inter-molecular disulfide bond, which does not involve C121, as this residue is supposed to induce the thiol/disulfide exchange reactions with the most likely disulfide bond C66–C160, which is found on the outer surface of β-Lg [3]. The most plausible scenario is the formation of an inter-molecular disulfide bond between C121 and C66; leading to the accessibility of a new reactive thiol group at C160. Probably the presence of the inter-molecular disulfide bond be-

Table 2
Peptides present in the in-gel tryptic digest of the disulfide-bound β-Lg dimer identified by MALDI–TOF MS

Peak no.	Fragment	Sequence	$(M+H)^+$	
			Obs.	Calc
1	15–20	VAGTWY	696.240	696.328
2	84–91	IDALNENK	916.464	916.466
3	92–100	VLVLDTDYK	1065.558	1065.575
4	92–101	VLVLDTDYKK	1193.662	1193.670
5	125–138	TPEVDDEALEKFDK	1635.776	1635.768
6	149–162	LSFNPTQLEEQCHI (−)	1658.786	1658.777
7	125–138 (+K)	TPEVDDEALEKFDK (+K)	1673.744	1673.768
8	21–40	SLAMAASDISLLDAQSAPLR	2030.068	2030.051
9	21–40 (+16)	SLAMAASDISLLDAQSAPLR (+16)	2046.039	2046.051
10	41–60	VYVEELKPTPEGDLILLQK	2313.252	2313.252
11	102–124 (var. B)[a]	YLLFCMENSAEPEQSLACQCLVR	2645.189	2645.179
12	102–124 (var. A)[a]	YLLFCMENSAEPEQSLVCQCLVR	2673.215	2673.211
13	15–40	VAGTWYSLAMAASDISLLDAQSAPLR	2707.369	2707.369
14	(61–69)=(149–162) (var. B)[b]	(WENGECAQK)=(LSFNPTQLEEQCHI)	2720.168	2720.191
15	(61–69)=(149–162) (var. A)[b]	(WENDECAQK)=(LSFNPTQLEEQCHI)	2778.192	2778.206
16	(61–70)=(149–162) (var. B)[b]	(WENGECAQKK)=(LSFNPTQLEEQCHI)	2848.267	2848.296
17	(61–70)=(149–162) (var. A)[b]	(WENDECAQKK)=(LSFNPTQLEEQCHI)	2906.259	2906.288
18	(149–162)=(149–162)[c]	(LSFNPTQLEEQCHI)=(LSFNPTQLEEQCHI)	3314.459	3314.554
19	(41–69) (var. A)	VYVEELKPTPEGDLEILLQKWENDECAQK	3416.629	3416.686
20	(41–70) (var. B)	VYVEELKPTPEGDLEILLQKWENGECAQKK	3486.601	3486.775
21	(41–70) (var. A)	VYVEELKPTPEGDLEILLQKWENDECAQKK	3544.671	3544.781
22	(61–69)=(102–124) (var. B)[c]	(WENGECAQK)=(YLLFCMENSAEPEQSLACQCLVR)	3706.575	3706.634
23	(61–69)=(102–124) (var. B/A)[c]	(WENGECAQK)=(YLLFCMENSAEPEQSLVCQCLVR)	3734.529	2734.666
24	(61–69)=(102–124) (var. A/B)[c]	(WENDECAQK)=(YLLFCMENSAEPEQSLACQCLVR)	3764.486	3764.635
25	(61–69)=(102–124) (var. A)[c]	(WENDECAQK)=(YLLFCMENSAEPEQSLVCQCLVR)	3792.570	3792.666
26	(61–70)=(102–124) (var. B)[c]	(WENGECAQKK)=(YLLFCMENSAEPEQSLACQCLVR)	3834.583	3834.729
27	(61–70)=(102–124) (var. B/A)[c]	(WENGECAQK)=(YLLFCMENSAEPEQSLVCQCLVR)	3862.670	3862.761
28	(61–70)=(102–124) (var. A/B)[c]	(WENDECAQK)=(YLLFCMENSAEPEQSLACQCLVR)	3892.669	2892.735
29	(61–70)=(102–124) (var. A)[c]	(WENDECAQKK)=(YLLFCMENSAEPEQSLVCQCLVR)	3920.643	3920.766
30	(102–124)=(149–162) (var. B)[c]	(YLLFCMENSAEPEQSLACQCLVR)=(LSFNPTQLEEQCHI)	4300.734	4300.972
31	(102–124)=(149–162) (var. A)[c]	(YLLFCMENSAEPEQSLVCQCLVR)=(LSFNPTQLEEQCHI)	4328.749	4329.003
32	(101–124)=(149–162) (var. B)[c]	(YLLFCMENSAEPEQSLACQCLVR)=(LSFNPTQLEEQCHI)	4428.802	4429.067
33	(101–124)=(149–162) (var. A)[c]	(YLLFCMENSAEPEQSLVCQCLVR)=(LSFNPTQLEEQCHI)	4456.812	4457.098
34	(41–69)=(149–162) (var. B)[b]	(VYVEELKPTPEGDLEILLQKWENGECAQK)=(LSFNPTQLEEQCHI)	5014.345	5014.457
35	(41–69)=(149–162) (var. A)[b]	(VYVEELKPTPEGDLEILLQKWENDECAQK)=(LSFNPTQLEEQCHI)	5072.359	5072.463
36	(41–70)=(149–162) (var. B)[b]	(VYVEELKPTPEGDLEILLQKWENGECAQKK)=(LSFNPTQLEEQCHI)	5143.483	5143.552
37	(41–70)=(149–162) (var. A)[b]	(VYVEELKPTPEGDLEILLQKWENDCAQK)=(LSFNPTQLEEQCHI)	5200.460	5200.558

[a] Peptides containing one fragment with an intra-molecular disulfide bond (=).
[b] Peptides containing two fragments linked by an intra- or inter-molecular disulfide bond (=).
[c] Peptides containing two fragments with an inter-molecular disulfide bond (=).

tween C160 and C160 in the non-native covalent dimer (Fig. 4B) points to the presence of dimers linked through two disulfide bonds (C121–C66; C160–C160) between two monomeric units. In the mass spectra of dimer and trimer hydrolysates, no peptide corresponding to an inter-molecular bond between C66 and C66, could be detected.

The mechanism of covalent bond formation during the heat-induced aggregation of β-Lg appears to be driven by the reactivity of only three cysteines: C121, C66, and C160 forming inter-molecular disulfide bonds. The different reactivities of the various cysteines can be explained by their different accessibilities in the molecular conformation adopted by the protein under the experimental conditions used.

Based on the results described, the initial stage of

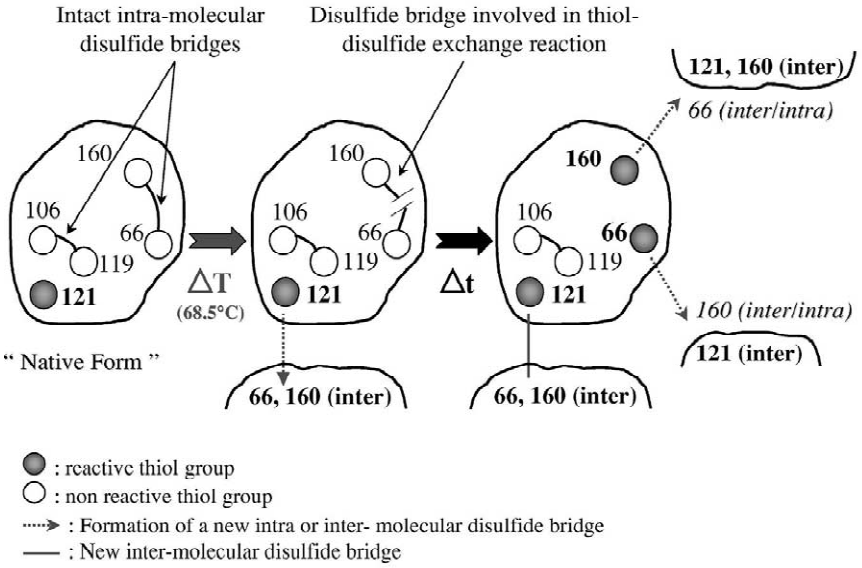

Fig. 5. Schematic representation of the different positions of heat-induced β-Lg disulfide crosslinks formed in the early stage of aggregation. ΔT = elevation of temperature (thermal activation); Δt = time separation between thermal activation and polymerization.

heat-induced aggregation of β-Lg under neutral and alkaline conditions can be schematically represented as shown in Fig. 5. It is hypothesized that the inter-molecular disulfide bonds initiating aggregate or gel formation implicate mainly C121, C66 and C160. In the first step, the reactive thiol group at C121 can react with the C66–C160 disulfide bridge of another β-Lg molecule then forming an inter-molecular C121–C160 or C121–C66 bridge. Successive reactions may involve C121, C160 or C66 from the dimer to react with disulfide bridges in other mono- or oligomers, forming higher oligomers.

Each monomeric β-Lg can potentially form up to three inter-molecular disulfide bonds. The formation of two inter-molecular disulfide bonds (e.g. C121–C66 and C160–C160) between two and the same β-Lg must be considered as an important factor limiting the process of network propagation.

4. Conclusion

In this study, we used a combination of SDS–PAGE with MALDI–TOF MS to obtain a clearer picture of the early stages of the heat-induced aggregation of β-Lg. In-gel tryptic digestions of heat-induced non-native monomer, covalent dimer and trimer, followed by MALDI–TOF analysis appeared to be a powerful tool to investigate the heat-induced aggregation of β-Lg at the molecular level.

In studying thiol/disulfide exchange processes in proteins, e.g. by SDS–PAGE or tryptic digestion, care should be taken to avoid artifacts that can occur at alkaline pH conditions

Our results revealed that heat-induced covalent aggregation of β-Lg at 68.5 °C, pH 6.7 is driven by thiol/disulfide exchange reactions involving mainly the three cysteines C121, C66, and C160.

The formation of a second disulfide bond (C160–C160) between the two monomer units of the covalent dimer gave us a strong indication that the thiol catalyzed mechanism does not end up necessarily in the formation of a linear aggregate with only one disulfide bond between each subunit. A second disulfide bond would be a limitation for the β-Lg-network propagation by restricting the number of possible orientations.

Heating β-Lg solutions at pH 4.9 does not result in any inter-molecular disulfide crosslinking and consequently, does not contribute to acidified milk gel formation.

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