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Formation of non-native β-lactoglobulin during heat-induced denaturation

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ABSTRACT

A mechanism describing the denaturation and aggregation behavior during heat-treatment of pure β-lactoglobulin and β-lactoglobulin in whey protein isolate (WPI) under selected conditions (20 to 90 gL^{-1} in water at pH 7.0, 78 ºC) is presented. A combination of reversed-phase and gel permeation chromatography was used to study the disappearance of native β-lactoglobulin and the formation of non-native intermediates in the aggregation process. The mean reaction order for pure β-lactoglobulin and β-lactoglobulin in WPI were the same, 1.4. While the rate of β-lactoglobulin denaturation was greater in WPI there was less aggregation compared to that of pure β-lactoglobulin. More of the β-lactoglobulin in WPI remained in a non-native monomer intermediate state after 30 min of heating. After an initial lag period, during which non-native monomers appeared, aggregates formed and rapidly reached a plateau in terms of their size. These aggregates were visualized using atomic force microscopy. There was no significant effect of protein concentration on either aggregate size or the number of exposed sulfhydryls in the heated solutions.

KEYWORDS

Whey proteins, denaturation/aggregation kinetics, thiols, stable intermediates.

INTRODUCTION

The heating and denaturation of whey proteins is an important process in conferring functional properties on them. As the proteins are heated, they unfold and form soluble aggregates. If heating is continued, then the aggregates start to form a gel

network. The aggregation occurs via covalent intermolecular disulfide bonds and non-covalent interactions such as van der Waals, ionic and hydrophobic interactions. Factors such as protein concentration, pH, salt and ionic strength can affect the formation of the gel network.

It has been shown previously that the number of free thiol groups rather than the size of the aggregates was key in determining the characteristics of whey protein gels[1]. As there are no exposed sulfhydryls in native β-lactoglobulin, the kinetics of β-lactoglobulin unfolding and aggregation is important for determining the characteristics of cold-set whey protein gels.

The kinetics of denaturation of β-lactoglobulin has been extensively studied using a wide variety of conditions, e.g. in whey protein isolates, milks, milk salts, various pH's etc. Roefs and de Kruif[2] put forward the theory that upon heating β-lactoglobulin, the denaturation kinetics is analogous to a radical addition polymerization reaction. Using concentrations of β-lactoglobulin between 2 gL^{-1} and 95 gL^{-1} at pH 6.75-6.95 they found that β-lactoglobulin denaturation closely fitted the predicted curves with a reaction order of 1.5. Dannenberg and Kessler[3] found β-lactoglobulin has a reaction order of 1.5 in skim milk, while a recent study by Claeys et al.[4] found that β-lactoglobulin denaturation followed first order kinetics in heated milk. Croguennec, O'Kennedy & Mehra[5] reported higher reaction orders, 1.96 and 1.98 for β-lactoglobulin A and B, respectively. They also reported that the presence or absence of NaCl or $CaCl_2$ altered the reaction order. Oldfield, Singh & Taylor[6] found that β-lactoglobulin denaturation in whey protein-depleted milks had a higher reaction order than in whey protein-enriched milks. When β-lactoglobulin was denatured in the presence of $CaCl_2$ at pH 7, the rate-limiting step was the aggregation step, resulting in an overall second-order reaction.

The initial step in the denaturation/aggregation pathway of β-lactoglobulin is the unfolding of β-lactoglobulin and the formation of a non-native monomer. The presence, after heating, of a monomeric species which has different characteristics to the native β-lactoglobulin was observed previously[7]. Croguennec et al. showed that the rearrangement of the disulfide bonds in β-lactoglobulin occurred causing the formation of non-native monomeric protein[8].

The reaction kinetics of β-lactoglobulin is greatly dependent on the temperature at which the protein is heated. There is a bend in the Arrhenius plot at ~90°C; below this temperature the rate of β-lactoglobulin unfolding limits aggregation reactions, at higher temperatures the β-lactoglobulin rapidly unfolds and the aggregation step is rate limiting[9]. Using DSC the same study showed the endotherm for β-lactoglobulin unfolding was centered between 75 and 80°C.

Many of the functionalities (in particular thickening and gelation) of whey proteins are dependent on the denaturation and aggregation of the protein, the role and importance of disulfide bonds in the aggregation and gelation processes is still debated. Hoffmann and Van Mil[10] showed that aggregate formation in β-lactoglobulin, heated at 65ºC, occurred mainly by covalent disulfide linkages. However, a recent study showed that blocking the free sulfhydryl groups present did not alter the rate of protein denaturation at β-lactoglobulin concentrations of 60 gL^{-1}, but at 10 gL^{-1} protein the rate of denaturation significantly decreased when the sulfhydryl groups were blocked[11]. The same paper reported no significant effect on the strength of gel formed by blocking the sulfhydryl groups before heating. The addition of BSA, a protein containing 17 disulfide bonds and one free sulfhydryl group, to β-lactoglobulin affected the order and rate of β-lactoglobulin denaturation.

BSA was also shown to have a detrimental effect on gel strength under certain conditions[12].

As β-lactoglobulin has no exposed sulfhydryl in its native state the unfolding of the protein and subsequent rearrangement of the disulfide bonds giving rise to a species with an exposed sulfhydryl may be the most important step in β-lactoglobulin functionality. This study examines the quantities of this non-native monomeric species in β-lactoglobulin and WPI solutions as a function of heating time and protein concentration.

MATERIALS AND METHODS

β-Lactoglobulin

Pure β-lactoglobulin was prepared in house (Moorepark Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland) by a modification of the method developed by Pearce[13]. Instead of whey a 12% (w/v) solution of WPC 70 (Lakeland Dairies, Ireland) was used as the starting material for the purification process. The β-lactoglobulin fraction was subjected to diafiltration and concentration using a 5,000 Da ultrafiltration membrane (Millipore). The protein was freeze-dried rather than spray dried to minimize any denaturation of the proteins. The powder was 88% protein by Kjeldahl. Prior to use the powder was resolubilized in Milli-Q water, adjusted to pH 4.6 and centrifuged at 32,000g to remove denatured protein. The pH of the protein solutions was adjusted to pH 7.0 with NaOH and extensively dialyzed (MWCO 8000 Da) against water to standardize the ionic strength. The conductivity of the dialyzed protein solutions was 500-700 μS/cm at a concentration of 50 gL^{-1}. After dialysis the protein concentration was determined by reversed-phase HPLC, using a Source^{TM} 5RPC (150×4.6 mm) column (Amersham Biosciences), 0.1% TFA in water

(solvent A) and 90% Acetonitrile 0.1% TFA (solvent B) were used for the separation. The following gradient was used to elute the proteins: 0-3 min 40% B, increased to 52% over 16 min, increased to 70% over 3 min, increased to 100% over 1 min and held for 5 min. A flow rate of 1 mL min^{-1} was used. The HPLC system consisted of a Waters 2695 separation module and a Waters 2487 dual wavelength absorbance detector. The data was acquired and processed using Waters Empower® software. β-Lactoglobulin, α-lactalbumin and BSA standards (Sigma Aldrich, Ireland) were used to calibrate the method. The purified protein preparations contained > 98% β-lactoglobulin and < 2% α-lactalbumin when analyzed by this method.

WPI

Whey Protein Isolate was obtained from Davisco Foods International, (Le Sueur, MN). A solution of the WPI was prepared, adjusted to pH 4.6 and centrifuged at 32,000 g to remove denatured material. The supernatant was adjusted to pH 7.0, freeze-dried and stored in the freezer until used. The powder contained 92.6% protein by Kjeldahl. Prior to use, the WPI was extensively dialyzed against water and the protein content measured using the RP-HPLC method described in the previous section. The protein content of the powder was 85% β-lactoglobulin and 15% α-lactalbumin.

Kinetics of denaturation of β-lactoglobulin

Protein solutions, ranging in concentration from 20 to 90 gL^{-1}, were filtered through a 0.22 μm syringe filter and heated in a water-bath at 78°C in sealed screw-top test tubes. Aliquots of 100 μL were removed at time 0, 1, 2, 5, 10, 15, 20, and 30 min and cooled on ice. The aliquots were diluted in an acetic acid buffer pH 4.6 and centrifuged at 20,000g for 20 min. The native β-lactoglobulin remaining in the

supernatant was quantified by reversed-phase HPLC using the method outlined above. The denaturation rate, k, of β-lactoglobulin is related to the reaction order, n by the following equation,

$dC/dt = kC^n$,

where C is the concentration of native protein. This equation is integrated to give,

$(C_t/C_0)^{1-n} = 1+(n-1)kt$ (for n>1),

where C_t is the native β-lactoglobulin concentration at time t and C_0 is the initial β-lactoglobulin concentration. Further rearranging gives,

$C_t/C_0 = [1+(n-1)kt]^{1/1-n}$.

The natural log of this equation was taken,

$\ln(C_t/C_0) = [1/(1-n)]\ln[1+(n-1)kt]$. (Equation 1)

Logging decay data such as this equalizes the data per unit time and reduces error when solving the equations. The reaction orders and rates were determined by fitting the experimental data to Equation 1. All experimental points were included in the curve fit; this is reasonable considering the rapid heating-up time of the protein solution, the solutions had reached 78ºC within one min. Introducing a lag-time to the data did not significantly alter the results obtained. The data were also fitted to the first order decay equation,

$\ln(C_t/C_0) = -kt$ (Equation 2)

to test for the possibility of first order kinetics.

Dynamic light scattering (DLS)

Protein solutions (β-lactoglobulin or WPI) were heated and aliquots removed at time intervals as described above. All samples were diluted to a concentration of 10 gL^{-1} protein using Milli-Q water prior to analysis. The hydrodynamic diameter of the

aggregates was determined using a Zetasizer Nano system (Malvern Instruments Inc., Worcester, UK). The measurements were carried out at 25ºC using a scattering angle of 12º. The cumulative method was used to find the mean size of a particle that corresponded to the mean of the volume distribution. The viscosity of the solvent was assumed to be the same as water, given the low concentration of protein.

Ellman's Assay

To determine the amount of exposed sulfhydryl groups in the heated β-lactoglobulin solutions, a variation of the assay developed by Ellman[10, 14] was used. The protein solutions were diluted to a concentration of 0.5 gL^{-1} protein in a 50mM Tris-HCl buffer pH 7.0. 2.75 mL of the diluted solution was placed in a cuvette and 0.25 mL of a 1 gL^{-1} 5,5'-dithio-bis(2-nitrobenzoic acid) (DTND) solution in 50mM Tris-HCl buffer was added. The solutions were incubated at room temperature for 30 min prior to reading the absorbance at 412nm. The molar extinction coefficient of free TNB, 14,150 $M^{-1}cm^{-1}$, was used to calculate the concentration of the sulfhydryl groups[15].

Gel permeation chromatography

Gel permeation chromatography was used to follow the formation of aggregates in the proteins solutions during heating. Heated samples were injected, 20 μL of 0.5 gL^{-1} protein, on a TSK G2000 column (TosoHaas, Montogomeryville, PA, USA) using a 50mM Tris-HCl buffer pH 7.0, at a flow rate of 1mL min^{-1}, as the eluent. The HPLC system described above was used for the separation.

Calculation of non-native monomers

The difference in the concentration of monomeric protein determined by gel permeation chromatography and native protein determined by precipitation at pH 4.6 and reversed-phase chromatography was attributed to the presence of non-native

monomers. As outlined in the introduction these species had been identified previously but not been quantified.

Atomic Force Microscopy

The native and heated β-lactoglobulin/WPI was imaged by atomic force microscopy, using an Asylum Research MFP-3DAFM (Asylum Research UK Ltd., Oxford, UK) in AC-mode. All samples were deposited undiluted onto a freshly cleaved mica surface and subsequently dried in a desiccator. An Aluminum reflex coated cantilever with a tetrahedral tip (AC 240), spring constant of 1.8 Nm^{-1} (Olympus Optical Co. Ltd, Tokyo, Japan), working frequency of 50-90 kHz, and scan rate of 1 Hz was used. The radius of curvature of the tetrahedral tip was 10 (± 3) nm. Images were processed using AFM imaging software Igor X.Y and Argyle Light for 3D images.

Statistical analysis

The denaturation kinetics experiments were carried out in triplicate. Each set of experimental data was fitted according to Equation 1, to determine the reaction order and rate for β-lactoglobulin denaturation. Using the three sets of results, an average value and standard deviation was calculated for each protein concentration. Analysis of variance (ANOVA) was carried out using Sigmastat (version 3.0; Jandel Scientific, Corte Madera, CA, USA). Student-Newman-Keuls method, pairwise multiple comparison procedures were used to determine differences between treatment means. For the Ellman assay and the DLS analysis, the experiments were carried out in duplicate. However, each measurement was made in triplicate. GPC measurements were carried out in duplicates. The results were subject to the same statistical analysis outlined above.

RESULTS

Results of the heat-denaturation (after 30 min) and subsequent gelation are summarized in Table 1.

β-lactoglobulin in H_2O

The amount of denatured β-lactoglobulin (at pH 7.0, 30 min at 78°C) increased with protein concentration. At 20 gL^{-1} β-lactoglobulin only 61% of the protein was denatured; at 90 gL^{-1} all of the protein was denatured. The data showed a poor fit for the first order kinetics equation except in the case of 90 gL^{-1} β-lactoglobulin, which fitted well to first order kinetics also. Overall the mean reaction order for the remaining concentrations was 1.4 ± 0.2.

The rate of β-lactoglobulin denaturation under the same conditions increased significantly with protein concentration, from $0.77 \pm 0.23 \times 10^{-3}$ to $4.71 \pm 0.09 \times 10^{-3}$ $(gL^{-1})^{1-n}s^{-1}$ over the concentration range studied (Table 1). There was a linear relationship between the rate of β-lactoglobulin denaturation and the initial β-lactoglobulin concentration.

Upon heating the whey proteins began to form soluble aggregates, which were monitored by dynamic light scattering (DLS) and by gel permeation chromatography (GPC). Protein concentration had no significant effect on the size of the aggregates formed in the solutions. At time zero the native β-lactoglobulin molecules were measured at 1.45 ± 0.14 nm in diameter. This was significantly smaller than the previously reported values of 4.08 nm in solution (at 0.1M ionic strength)[16] or x-ray crystallography[17]. The lower value may be because of the low ionic strength of the system used, when β-lactoglobulin was measured in milk salts a hydrodynamic diameter closer to those reported in the literature was found, 4.3 to 4.8 nm. It should be noted that the measurements by DLS were at the lower limit of detection for the instrument used in this study. Therefore, values in the single digit nanometer range

should be treated with caution. Figure 1. shows AFM images of the native β-lactoglobulin prior to heating; the height profile shows a protein size of approximately 0.8 to 1.5 nm. Though due to the tight packing of the protein only a portion of the protein molecule may detectable by AFM; so the 1.5 nm may be closer to a radius value than a diameter measurement.

The mean size of the aggregates after 30 min heating was 21.5 ± 3.3 nm, which is substantially smaller compared to the reported particle sizes of heated β-lactoglobulin in the literature. However, it should be noted that the starting material used in this study was practically free of any denatured and aggregated material. Preliminary experiments (data not shown) revealed significantly larger aggregates if min amounts of denatured or aggregated material was present in the starting material. There was a lag period longer than that of the heating delay (see Material and Methods) before aggregation occurred. The first aggregates were observed to form between three and ten min. The lag time was shorter in solutions containing higher protein concentrations (Figure 2). The size of the aggregates reached a plateau at these times and there was no significant change in the size of the aggregates during further heating. This agrees with a recent study where the formation of aggregates was not observed until seven min of heating had elapsed, the size of the aggregates obtained also compared well with the present study[18].

Gel permeation chromatography (Figure 3) showed a decrease in monomer and an increase in aggregated β-lactoglobulin during heat-treatment. Integration of the monomeric peak gave the concentration of β-lactoglobulin monomers. The amount of aggregation was calculated by subtraction from the initial protein concentration. The GPC results agreed with those of DLS; only little aggregation was detected during the first five min of heating, after which an increase in aggregation was observed. The

amount of aggregates present after 30 min of heating increased logarithmically with protein concentration, accordingly, the amount of monomer decreased exponentially. Comparing reversed-phase and GPC-HPLC allowed for the quantification of non-native monomers, some of which have been described previously[8, 19]. Essentially these species were formed by the first step of the aggregation mechanism, whereby some irreversible unfolding and/or intramolecular disulfide interchange took place, which gave rise to a non-native monomer. Previous studies had established the presence of these species but had not quantified the amounts present. The non-native monomer concentration initially increased as a function of heating time and then decreased once aggregation commenced (Figure 4.). The amount of non-native monomer present after 30 min decreased with increasing protein concentration (Table 1.).

As discussed in the introduction, the formation of intermolecular disulfide bonds is thought to be critical in the aggregation process. In fact the kinetic model put forward by Roefs and de Kruif[2] was based on the polymerization occurring through the formation of disulfide bonds. For this reason the exposure of sulfhydryl groups as a function of heating time was determined by the Ellman assay. The concentration of the exposed sulfhydryl increased logarithmically with heating time. The concentration of exposed sulfhydryl present in the samples after 30 min of heating was independent of protein concentration under the conditions used for this study (Table 1).

β-lactoglobulin denaturation in WPI at pH 7.0

The α-lactalbumin protein was rapidly denatured upon heating. There was no native α-lactalbumin detected (RP-HPLC) after 15 min of heating for any sample.

β-lactoglobulin denaturation in WPI was faster than in pure β-lactoglobulin systems. The rate of denaturation increased significantly ($P \geq 0.05$) from $1.58 \pm 0.12 \times 10^{-3}$ to $10.61 \pm 0.26 \times 10^{-3}$ $(gL^{-1})^{1-n}s^{-1}$ as the protein concentration was increased from 20 to 90 gL^{-1}. The percentage of denatured β-lactoglobulin was greater in WPI than in pure β-lactoglobulin. More than 80% of the β-lactoglobulin was denatured after heating for 30min regardless of the initial protein concentration.

The order for β-lactoglobulin denaturation in WPI under the conditions used in this study was 1.4 ± 0.1, the same as for pure β-lactoglobulin. There was no relationship between order and protein concentration over the range studied (Table 1).

The mean aggregate size after 30 min heating was 23.7 ± 4.3 nm, the same size as the aggregates formed in pure β-lactoglobulin solutions. There was a shorter lag period, compared to that in pure β-lactoglobulin, before aggregate formation started (Figure 2). The onset of aggregate formation occurred between two and three min. The gel permeation results were more difficult to interpret in the case of WPI than for pure β-lactoglobulin; the reason for this is that monomeric β-lactoglobulin (18 kDa) and α-lactalbumin (14 kDa) co-elute on the GPC column. A double peak could be observed in the unheated WPI GPC profile (Figure 3 B). As the sample is heated the double peak changed into a broad monomer peak, where the right hand shoulder of the peak (due to the presence of α-lactalbumin) decreased at a faster rate than the β-lactoglobulin peak on the left of the monomer peak. The quantity of aggregate present after 30 min of heating increased with increasing protein concentration (Table 1). The amount aggregated was generally lower than that in pure β-lactoglobulin. The concentration of non-native monomer was calculated as described above. However, the presence of monomeric α-lactalbumin can effect the results. As mentioned above, α-lactalbumin is denatured faster than β-lactoglobulin. When α-lactalbumin was heat

denatured in the presence of β-lactoglobulin it formed disulphide linked homopolymers and heteropolymers with β-lactoglobulin[20, 21]. After 30 min of heating α-lactalbumin may have only contributed a small amount to the monomer peak in the heat denatured WPI samples because, originally only 15% of the WPI was α-lactalbumin, it denatured rapidly and it was likely to have aggregated. The amount of non-native monomer generally decreased as a function of protein concentration. At protein concentrations from 20 to 80 gL^{-1} the amount of non-native monomer present after 30 min was significantly higher in WPI than in pure β-lactoglobulin. There was also more non-native monomer present in the WPI solutions as a function of time than in the pure β-lactoglobulin solutions (Figure 4).

The AFM image in Figure 5 shows an example of a protein aggregate formed in a 50 gL^{-1} WPI solution heated for 30 min. The height of the aggregate was approximately 35 nm, which is somewhat larger than the particle size of 22 ± 2 nm determined by dynamic light scattering. However, it is interesting to note that the aggregate is surrounded by seemingly monomeric proteins; this could be either some of the remaining native protein (6 ± 1 %) or non native monomers (17 ± 1 %). DLS could not distinguish between monomers and aggregates of that size, however, the overall mean diameter will appear smaller, which could explain the above discrepancy.

When the concentration of exposed sulfhydryl was calculated, only the concentration of β-lactoglobulin in the samples was considered in the calculations as α-lactalbumin has no free sulfhydryl. Sulfhydryl exposure was independent of protein concentration as was the case in pure β-lactoglobulin (Table 1.). Like pure β-lactoglobulin, the increase in exposed sulfhydryl in WPI was logarithmic.

DISCUSSION

The conditions used in this study were chosen so as to maximize the quantity of β-lactoglobulin unfolding while limiting the rate at which aggregation was occurring. To this end the temperature of 78°C was used as it falls within the temperature range where unfolding is limiting the rate of the reaction; at this temperature the degree of unfolding of the protein has an intermediate value[9, 22]. Using low ionic strength and neutral pH also limits aggregation[23]. Finally removing any denatured material from the solutions, by using a precipitation step at pH 4.6, also slows the rate of aggregation.

The previously proposed mechanism predicted a reaction order of 1.5[2]. However, many authors reported different results when reaction orders were determined experimentally; in general values between first order and second order have been reported depending on the conditions used[5, 6, 11, 23, 24]. The mean reaction orders of pure β-lactoglobulin and β-lactoglobulin in WPI were 1.4 under the conditions used here. The mean reaction order in milk-ultrafiltrate was significantly higher, 2.2 ± 0.6 (unpublished data). It is clear from the results presented here that the presence of α-lactalbumin (as is the case in WPI) had no effect on the reaction order under the conditions used here. A previous study found that BSA did have a significant effect on reaction order[12]. These results could be significant in relation to the role played by disulphide bonds in the denaturation/aggregation process. BSA contains one free sulfhydryl group while α-lactalbumin has none. For this study only one heating temperature was used for the reasons outlined above. As mentioned in the introduction the kinetics of β-lactoglobulin denaturation is greatly dependent on the temperature of heating so heating at a higher or lower temperature could potentially give rise to different denaturation kinetics.

There were also significant differences between the rates of denaturation of pure β-lactoglobulin and of β-lactoglobulin in WPI; at the same protein concentrations the rate of denaturation was higher in the WPI. The reaction rates were on the same order of magnitude to those reported previously in pure β-lactoglobulin solutions[5, 11], the lower concentrations had similar reaction rates to those reported in skim milk at pH 7.0 (2.65×10^{-3} s^{-1})[25]. The WPI solutions had been extensively dialyzed and any denatured material removed prior to use, which means that the differences in reaction rate are likely to be due to the difference in the protein content, α-lactalbumin.

The RP-HPLC results only measured the disappearance of native β-lactoglobulin. In many of the previous studies only the disappearance of native β-lactoglobulin was studied. Comparing the results from RP-HPLC and GPC gave a new insight into the formation of the intermediates during the activation step of polymerization; the results show that non-native, monomeric intermediates are present in the heated solutions at significant concentrations under certain conditions. While previous studies had identified non-native, monomeric intermediates, they were generally not quantified.

There was still a large portion of the β-lactoglobulin remaining native or as non-native monomers after 30 min of heating, in the case of protein heated at lower concentrations. This indicates that while β-lactoglobulin unfolding and the formation of non-native monomers is occurring aggregation reactions are occurring at a slower rate so the non-native monomers are not being used in propagation reactions to form aggregates. In the WPI solutions the concentration of non-native monomer was greater, meaning that the difference between the rate of β-lactoglobulin unfolding and β-lactoglobulin aggregation was larger.

The role of α-lactalbumin in the denaturation/aggregation process is interesting; in the case outlined here α-lactalbumin increased the rate of β-lactoglobulin denaturation

without affecting the reaction order. Even though α-lactalbumin lacks a free sulfhydryl it has been shown to be involved in hetero- and homo-aggregates in the presence of β-lactoglobulin via disulphide interchange reactions.

DLS results showed that during the first few min of heating there was no propagation reactions. In agreement with this, the concentration of non-native monomer increased during this period. Once the propagation reactions were underway the aggregates rapidly reached a constant size. The size of the aggregates formed here were smaller than previously reported results. This could be because of the very pure protein used and the low ionic strength of the system. A more recent study found similar results to those reported here[18]. The presence of α-lactalbumin did not alter the size of the aggregates even though the rate of the reaction had increased. While the size of the aggregates rapidly reached a maximum, the number of aggregates increased with both heating time and protein concentration (determined by GPC).

The AFM analysis further supported the results obtained from gel permeation and light scattering. The image of the unheated β-lactoglobulin show a sea of monomers which are of the same height as those previously determined by x-ray crystallography and by dynamic light scattering here. A previous study has determined that drying the samples prior to AFM measurements did not effect the proteins when compared to measurements made in liquid[26]. The AFM image of the heated WPI solution shows an aggregate surrounded by material which is monomeric in height. This allows a visualization of what is observed in the gel permeation data. There are still significant quantities of monomer material remaining in solution with the aggregates.

The non-native monomers previously identified were brought about by a rearrangement of the disulphide bonds in native β-lactoglobulin leading to the sulfhydryl group on Cys119 being exposed and a disulphide bond forming between

Cys119 and Cys121[8]. As well as this another recent study has found monomeric β-lactoglobulin species where the other disulfide bond, Cys66-Cys160, has been cleaved to leave an exposed sulfhydryl group on one of the cysteines[27]. It has been suggested that these non-native monomers are less reactive than unfolded β-lactoglobulin with the native disulphide bonds intact; this hypothesis is supported in the results presented here by the presence of significant quantities of non-native monomer after heating. These non-native monomers behave differently to native β-lactoglobulin in that they are insoluble at pH 4.6 and elute slightly earlier in GPC chromatography than native β-lactoglobulin[8]. The role of these non-native monomers in the functionalities, such as gelation, of heated β-lactoglobulin solutions is poorly understood. Given the quantity of these non-native monomers present it seems that they are very stable and not reactive under these conditions. They may still play a role in the functionality of the whey proteins when the environmental conditions are altered, further heating or a drop in pH will cause rearrangements in these species there-by increasing their reactivity.

In conclusion the present study confirms that the previously identified non-native monomers can be formed in significant quantities during the heat denaturation of β-lactoglobulin.

ABBREVIATIONS USED

BSA	Bovine serum albumin
DTNB	5,5’-dithio-bis(2-nitrobenzoic acid)
G'	Elastic modulus
GDL	Glucono-δ-lactone
GPC	Gel permeation chromatography
PAGE	Polyacrylamide gel electrophoresis
RP-HPLC	Reversed-phase high-performance liquid chromatography
SH	Sulfhydryl group
WPI	Whey protein isolate

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FIGURE CAPTIONS

Table 1. Summary of results after 30 min of heating and subsequent gelation.

Figure 1. AFM images showing height (A), amplitude (B) and phase (C) data for native β-lactoglobulin. The graph at the bottom shows the height across the cross-section marked in panel A.

Figure 2. Representative sample of particle sizing results as determined by dynamic light scattering. (A) β-lactoglobulin (B) WPI. Protein concentrations 20 gL^{-1} ◆, 50 gL^{-1} ●, 90 gL^{-1} ▲.

Figure 3. Typical gel permeation profiles as a function of heating time. Samples heated for 0, 1, 2, 3, 5, 10, 15, 20 and 30 min at 78ºC (A) 80 gL^{-1} β-lactoglobulin (B) WPI 80 gL^{-1} protein.

Figure 4. Distribution of protein in (A) 20 gL^{-1} and 90 gL^{-1} β-lg and (B) 20 gL^{-1} and 90 gL^{-1} WPI solutions. The horizontal lines represent the proportion of native β-lactoglobulin as measured by RP-HPLC. The vertical lines represent non-native monomeric β-lactoglobulin (the difference in monomeric by GPC and native by RP-HPLC). The clear region was the quantity which was aggregated (measured by GPC).

Figure 5. Atomic Force Microscopy images (recorded in air) show height (A), amplitude (B) and phase (C) data for a WPI solution 50 gL^{-1} heated for 30

min at 78°C. A three dimensional representation (with an overlaid phase image) of the large aggregate is shown in the image to the right of panel A. The graph at the bottom shows the height profile across the cross-section marked in panel A.

Solution and protein conc.	Reaction order	Reaction rate $(gL^{-1})^{1-n}s^{-1} \times 10^{-3}$	% Denatured[a]	% Aggregated[b]	% non-native monomer[c]	Particle diameter (nm)	Mol exposed SH per mol protein
β-lg 20 gL^{-1}	1.6 ± 0.4	0.77 ± 0.23[z]	61 ± 4	49 ± 1	12 ± 5	25 ± 1	0.22 ± 0.01
β-lg 30 gL^{-1}	1.3 ± 0.1	1.11 ± 0.08[z]	78 ± 1	65 ± 1	13 ± 2	23 ± 1	0.19 ± 0.01
β-lg 40 gL^{-1}	1.4 ± 0.0	1.92 ± 0.07[zy]	88 ± 1	75 ± 1	13 ± 2	21 ± 1	0.27 ± 0.01
β-lg 50 gL^{-1}	1.4 ± 0.1	2.59 ± 0.30[y]	92 ± 0	83 ± 0	9 ± 0	20 ± 1	0.33 ± 0
β-lg 60 gL^{-1}	1.5 ± 0.2	3.73 ± 0.78[x]	94 ± 3	88 ± 0	6 ± 3	26 ± 0	0.31 ± 0
β-lg 70 gL^{-1}	1.3 ± 0.1	4.38 ± 0.65[x]	98 ± 0	94 ± 1	4 ± 1	19 ± 3	0.30 ± 0.02
β-lg 80 gL^{-1}	1.2 ± 0.1	4.38 ± 0.60[x]	99 ± 1	96 ± 0	3 ± 1	19 ± 2	0.30 ± 0.01
β-lg 90 gL^{-1}	1.0 ± 0	4.71 ± 0.09[x]	100 ± 1	97 ± 0	3 ± 1	17 ± 2	0.26 ± 0
[e]WPI 20 gL^{-1} (17 gL^{-1})	1.6 ± 0.1	1.58 ± 0.12[wv]	82 ± 1	19 ± 3	[f]63 ± 4	28 ± 11	0.26 ± 0.02
[e]WPI 30 gL^{-1} (26 gL^{-1})	1.4 ± 0.1	2.30 ± 0.13[wvt]	91 ± 1	48 ± 6	[f]43 ± 7	26 ± 1	0.35 ± 0
[e]WPI 40 gL^{-1} (35 gL^{-1})	1.4 ± 0.1	2.41 ± 0.30[vus]	90 ± 0	67 ± 7	[f]23 ± 7	24 ± 2	0.33 ± 0.05
[e]WPI 50 gL^{-1} (43 gL^{-1})	1.4 ± 0.0	2.98 ± 0.36[ts]	94 ± 1	73 ± 0	[f]21 ± 1	22 ± 2	0.38 ± 0.01
[e]WPI 60 gL^{-1} (52 gL^{-1})	1.3 ± 0.1	4.00 ± 0.43[r]	97 ± 1	69 ± 0	[f]28 ± 1	22 ± 2	0.34 ± 0
[e]WPI 70 gL^{-1} (60 gL^{-1})	1.2 ± 0.1	4.63 ± 0.33[r]	99 ± 1	82 ± 1	[f]17 ± 2	23 ± 1	0.31 ± 0.01
[e]WPI 80 gL^{-1} (69 gL^{-1})	1.4 ± 0.1	9.29 ± 1.17	99 ± 0	85 ± 2	[f]14 ± 2	23 ± 1	0.28 ± 0.03
[e]WPI 90 gL^{-1} (78 gL^{-1})	1.3 ± 0	10.61 ± 0.26	99 ± 1	96 ± 0	[f]3 ± 1	22 ± 1	0.35 ± 0.01

[a] Measured by gel reverse-phase HPLC
[b] Measured by Gel permeation chromatography
[c] % denatured - % aggregated
[e] Values in brackets was the concentration of β-lactoglobulin in the sample.
[f] Monomeric α-lactalbumin remaining in the WPI will alter the results for the amount of monomeric β-lactoglobulin remaining. The letters after the rate values indicate which values are the same statistically.

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565 **Table 1.**

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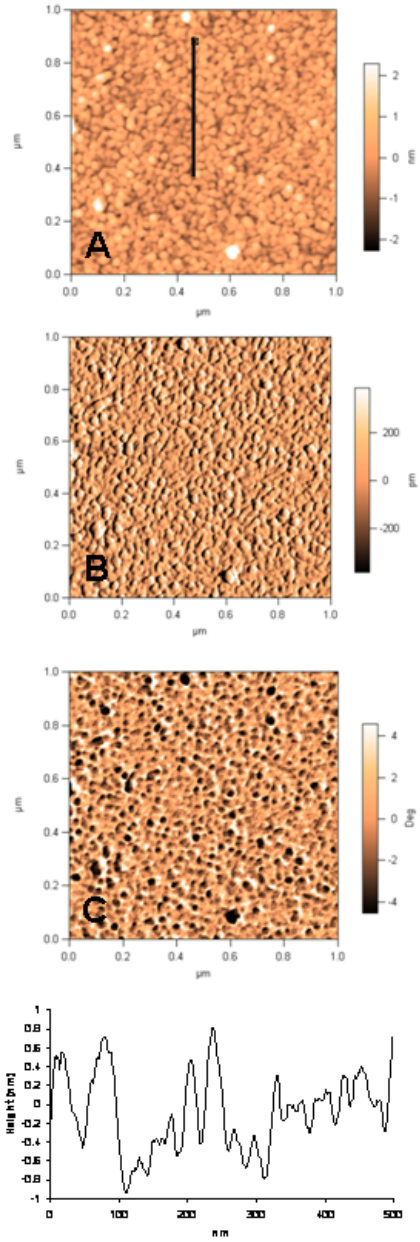

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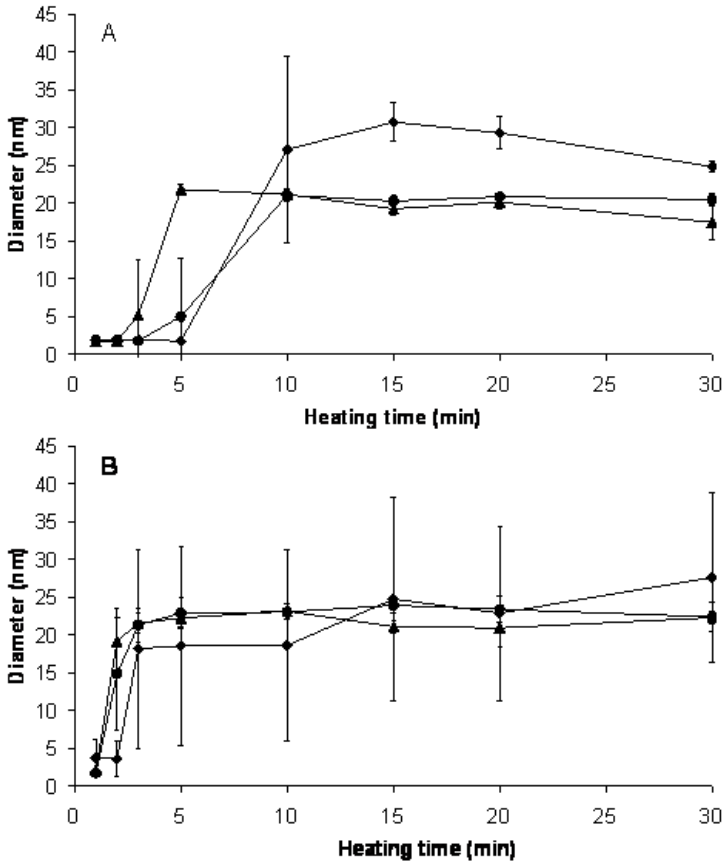
572 **Figure 1.**



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574 **Figure 2.**

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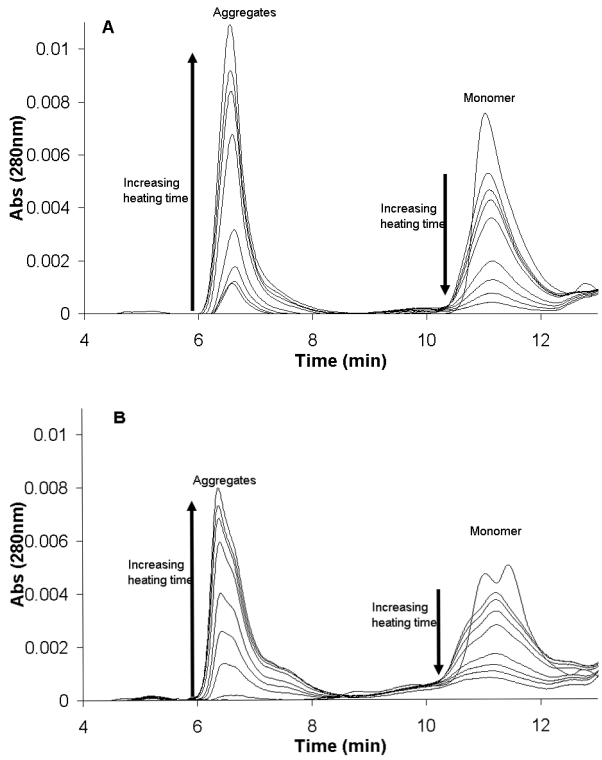

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578 **Figure 3.**

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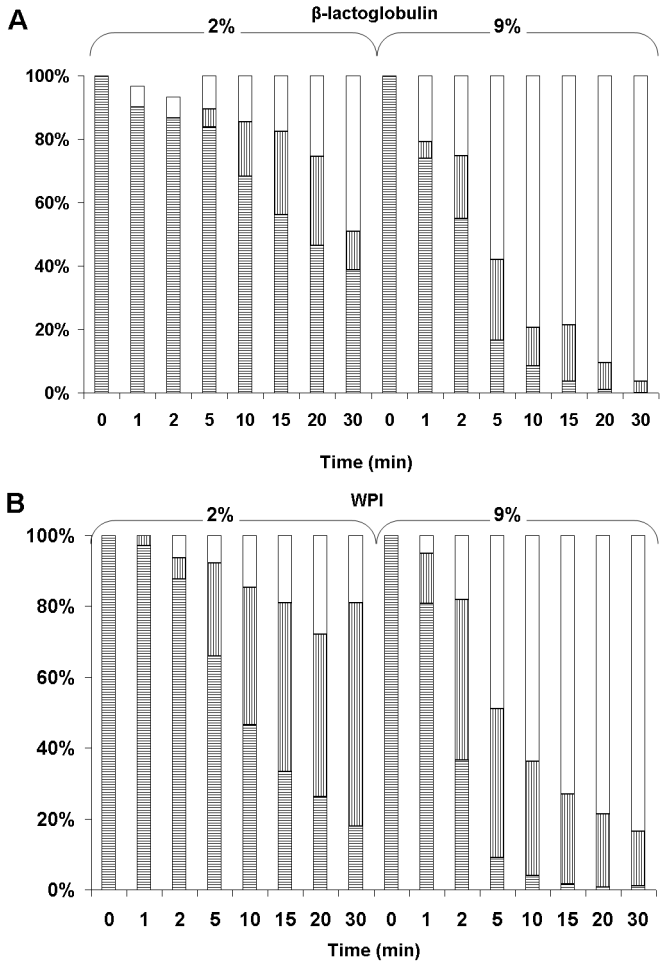

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582 **Figure 4.**

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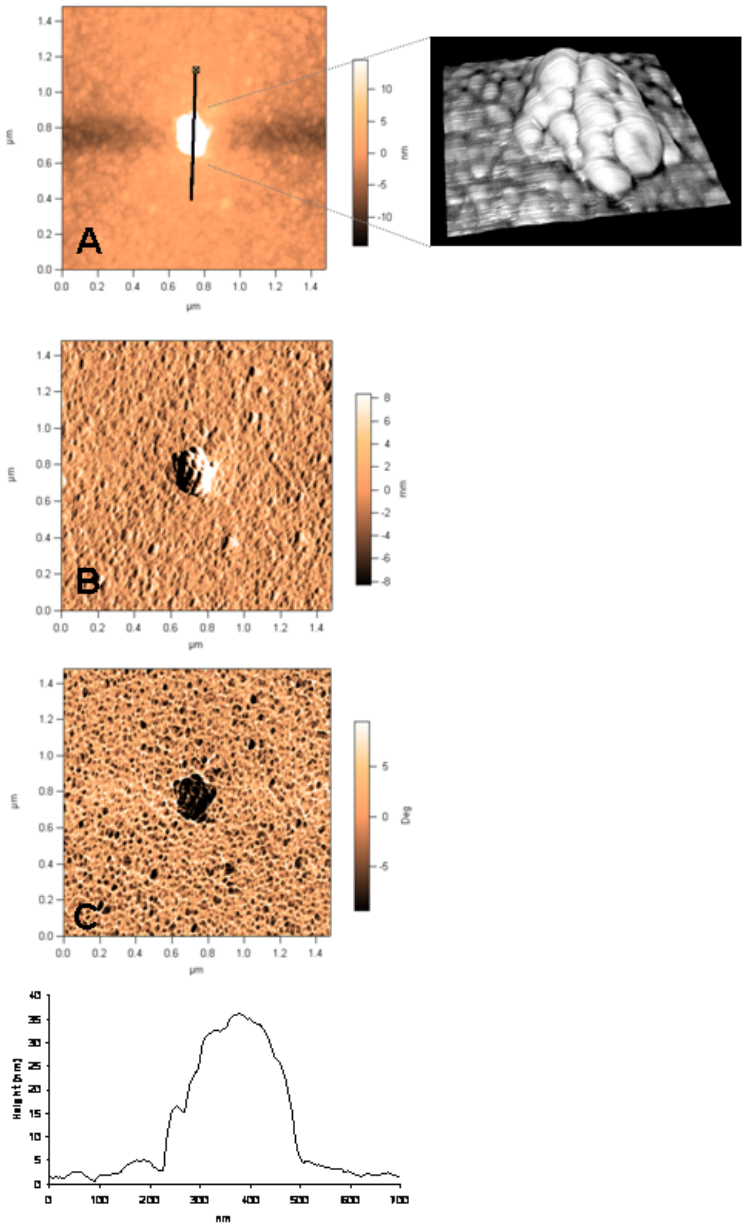

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585 **Figure 5.**

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