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The Influence of Bovine Serum Albumin on β-Lactoglobulin Denaturation, Aggregation and Gelation.

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Abstract

The effect of bovine serum albumin (BSA) on the heat induced denaturation, aggregation and subsequent acid-induced gelation of β-lactoglobulin (β-lg) was investigated in this work. Changes in the denaturation kinetics of β-lactoglobulin during heating at 78°C were determined by monitoring the disappearance of the native protein by reverse-phase chromatography. Replacing β-lactoglobulin with increasing amounts of BSA, while keeping the total protein concentration constant at 5% (w/w), significantly increased the denaturation rate of β–lactoglobulin from $2.57 \pm 0.30 \times 10^{-3} (gL^{-1})^{(1-n)} s^{-1}$ to $5.07 \pm 0.72 \times 10^{-3} (gL^{-1})^{(1-n)} s^{-1}$ (β-lg : BSA ratio of 3:1 w/w). The reaction order for β-lactoglobulin was 1.40±0.09. Partial replacement of β-lactoglobulin with BSA (β-lg : BSA ratio of 3:1 w/w) significantly increased the reaction order to 1.67±0.13. Heat-induced aggregates between β-lactoglobulin and BSA were studied by dynamic light scattering, two-dimensional electrophoresis and size exclusion chromatography. The partial replacement of β-lactoglobulin with BSA significantly changed the gelling properties of the acid-induced gels. A rapid rate of acidification resulted in a significant decrease, while a slow acidification rate resulted in a significant increase in gel strength. Size exclusion chromatography demonstrated that intermolecular disulphide bond formation occurred during both heat-induced denaturation/aggregation and subsequent acid-induced gelation. Results clearly indicate that BSA contributed to the formation of these disulphide bonds.

Keywords: β-lactoglobulin; denaturation; Bovine Serum Albumin; thiol groups; kinetics; gelation.

1. INTRODUCTION

β-Lactoglobulin (β-lg) is the major whey protein in bovine milk making up approximately 50% of the total whey protein. It has a molecular weight of 18kDa and contains two disulphide bonds and one free sulphydryl group. Bovine serum albumin (BSA) is a minor whey protein with a molecular weight of 66kDa. It contains 17 disulphide bonds and one free sulphydryl group. Previous studies have shown that the intermolecular disulphide bonds can be formed during both heat treatment and acid-induced gelation of whey protein mixtures (Matsudomi, Rector & Kinsella, 1991; Alting, Hamer, de Kruif & Visschers, 2000; Havea, Singh & Creamer, 2000; Havea, Singh & Creamer, 2001). Alting et al. (2000) investigated the cold gelation of whey protein isolate (WPI), where the whey proteins are initially heat-denatured and gelation was induced by slow acidification at room temperature. In this study, it was found that disulphide bonds are important for determining gel hardness. It was proposed that intermolecular disulphide bonds are formed during the later stages of gelation at lower pH's. Another study by the same authors (Alting, Hamer, de Kruif, Paques & Visschers, 2003) found that the size of the aggregates formed during the initial heating step was not critical to the final gel strength. It was, however, suggested that the number of free sulphydryl groups essentially determined the strength of the gel.

The interaction between BSA and β-lg in heat-induced gels has been studied previously (Tobitani & Ross-Murphy, 1997). It was found that the ratio of the two proteins to each other affected the gelation behaviour with the inclusion of BSA accelerating the formation of the heat-set gels. An earlier study (Hines & Foegeding,

1993) found that BSA increased the elastic modulus of heat induced gels when added to β-lg. It was also found that the rate of denaturation of BSA was much greater than that of β-lg when the proteins were mixed in an equimolar ratio (22 : 78 ratio of β-lg : BSA; w/w) in the presence of 100mM NaCl. Under the same conditions the addition of BSA increased the rate of denaturation of β-lg. In skim milk, different effects have been observed (Oldfield, Singh & Taylor, 2005). The rate of denaturation of β-lg in skim milk decreased at higher whey protein concentrations, whereas the rate of BSA denaturation increased under the same conditions. Gelation of β-lg/BSA mixtures have so far only been studied under thermally-induced conditions. Matsudomi et al. (1991) and Matsudomi, Oshita & Kobayashi (1994) found that the presence of BSA increased the gel strength in heat-set β-lg gels and stressed the importance of disulphide bonds in the gelation of BSA. Gezimati, Singh and Creamer (1996) studied heat-set gels formed with β-lg/BSA mixtures at 70°C and 75°C. BSA increased the gel strength both during heating and after cooling to 25°C. When heating β-lg/BSA mixtures at a rate of $1 Kmin^{-1}$, BSA aggregated and gelled before β-lg. Havea et al. (2000; 2001) demonstrated that BSA can form heat-induced intermolecular disulphide bonds with β-lg and α-lactalbumin (α-la).

The work presented in this paper examines the cold gelation behaviour of mixed β-lg/BSA systems. The heat-induced denaturation of β-lg in the presence of various BSA concentrations was studied in detail. The kinetic reaction order and denaturation rates are determined for β-lg. A possible mechanism for the denaturation and aggregation of the two proteins is proposed. The acid-induced gelation of these mixtures was studied to determine the role of BSA in gel formation of β-lg. Turbidity and rheological properties were measured during acidification. The formation of intermolecular disulphide bonds was monitored by comparing covalently linked

protein aggregates at different points during the cold gelation process, analysed by size exclusion chromatography.

2. MATERIALS AND METHODS

2.1 Materials

A highly enriched β-lg fraction was prepared in house using differential precipitation and subsequent ultrafiltration (Mehra, 2001). The protein was freeze-dried rather than spray dried to avoid any denaturation of the proteins (88% protein by Kjeldahl).

Bovine Serum Albumin (BSA) lot no. 021k7606 was obtained from Sigma Aldrich (Dublin, Ireland). The manufactures found the powder to be 97% pure, by agarose electrophoresis. All other chemicals and reagents used were purchased from Sigma Aldrich (Dublin, Ireland) unless otherwise stated.

2.2 Heat denaturation

Prior to each experiment, the powders were resolubilised in distilled water and adjusted to pH 7.0 using NaOH. Both protein solutions were dialysed against distilled water overnight (in the same solution) to standardise their ionic strength. After dialysis, the protein concentration was determined by reverse phase HPLC using a Source™ 5RPC column (Amersham Biosciences UK limited). The HPLC system consisted of a Waters 2695 separation module with a Waters 2487 dual wavelength absorbance detector. The method used for separation was described elsewhere (Croguennec, O'Kennedy & Mehra, 2004). The data were acquired and processed using Waters empower software (Milford, MA, USA).

The stock solutions of dialysed proteins were diluted to the appropriate concentration and filtered through a 0.22μm Acrodisc syringe filter (Pall Corporation, Ann Arbor, MI, USA). The solutions contained 5% total protein, four different ratios of β-lg : BSA were used, 100 : 0, 90 : 10, 80 : 20 and 75 : 25 (w/w). The solutions were heated in sealed screw top test-tubes at 78°C for 30 minutes in a water bath, after which the tubes were immediately cooled on ice. The tubes were 13.5 mm in internal diameter with 1.3 mm walls and contained 3mL of solution. The solution heated up rapidly and had reached 78°C within the first minute.

Using differential scanning calorimetry (DSC) Verheul, Roef and de Kruif (1998) reported a denaturation peak at temperatures around 80ºC for β-lg. A DSC study of BSA showed two denaturation peaks, one at 56ºC and the other at 69ºC. (Michnik, 2003). Both of the studies were carried out under conditions similar to those used in this study.

The **denaturation kinetics** of β-lg was studied in the presence of the four different BSA concentrations outlined above. Aliquots of the solutions were removed after 0, 1, 2, 5, 10, 15, 20 and 30 minutes of heating at 78°C and cooled immediately on ice. The denatured protein was removed by acid precipitation using an acetic acid buffer pH 4.6 followed by centrifugation at 20,000g for 20 minutes. This is similar to methods employed previously (Verheul et al., 1998). Ju and Kilara (1998) claimed that at pH 4.6 some native whey proteins formed aggregates. This did not occur in our work as no protein precipitated when the unheated, time 0, sample was adjusted to pH 4.6. The supernatant was diluted and the native β-lg concentration was determined using the HPLC method described above. The denaturation rate of β-lg is related to the reaction order by the following equation,

$dC/dt = kC^n$,

where k is the reaction rate, n is the reaction order and C the concentration of native protein (Kessler & Beyer, 1991). This equation can be integrated to give,

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

where C_t is the native β-lg concentration at time t, C_0 is the initial β-lg 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 equalises 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. 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 rule out the possibility of first order kinetics.

Dynamic light scattering (Malvern Zetamaster; model 7EM; Malvern Instruments Ltd, Worcester, UK) was used to measure the sizes of the aggregates formed during the initial heating step of cold gelation. The scattered light was detected at a fixed angle of 90°. The cumulative method was used to find the mean average (z-average) or the size of a particle that corresponded to the mean of the intensity distribution.

Two dimensional electrophoresis was carried out according the methods described previously (Laemmli, 1970; Havea, Singh, Creamer & Campanella, 1998).

The first dimension was carried out under denaturing but non-reducing conditions, a 4%-15% gradient gel was used (Bio-Rad, Alpha Technologies, Dublin, Ireland). The strips of gel from the first dimension were heated to 95°C in a solution containing 62.5mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulphate (SDS) and 5% β-mercaptoethanol. This caused the break-up of protein aggregates by reducing intra- and intermolecular disulphide bonds. The gel strip was placed on top of a separating gel consisting of 15% acrylamide and a 4% stacking gel, both containing 0.1% SDS. The electrophoresis was carried out using a constant voltage of 155V in a Mini Protean II system (Bio-Rad, Alpha Technologies, Dublin, Ireland). The gels were stained in a 0.5% Coomassie brilliant blue R-250, 25% isopropanol, 10% acetic acid solution.

High pressure size exclusion chromatography was used to study the disulphide linked aggregates. The aggregates formed during the heating stage were treated with a buffer comprising of 20mM Bis-tris pH 7.0, 5% SDS and 50mM iodoacetamide (IAA). The treated aggregates were stirred overnight at room temperature and filtered through a 0.45μm syringe filter prior to analysis. An ÄKTA Purifier chromatography system (Amersham Bioscience UK limited) with a TSK G2000 and a TSK G3000 columns (TosoHaas, Montogomeryville, PA. USA) in series were used for the separation. The eluent was 20mM sodium phosphate buffer at pH 7.0 containing 1% SDS. A dye, blue dextran 2000, was used to determine the void volume of columns.

2.3 Acid gelation

Heat denatured samples were diluted to a total protein concentration of 2%. A 0.5% or 0.15% concentration of glucono-δ-lactone (GDL) was added as a solid and

stirred to dissolve. The rheological measurements were carried out using a Bohlin CVO rheometer (Malvern instruments, Worcestershire, UK.). The measurements were carried at 25°C out using the bob and cup conformation with a frequency of 1Hz and an initial stress of 0.01235. The measurements were carried out over a five hour period for the samples containing 0.5% GDL and over 20 hours for the samples containing 0.15% GDL. The pH of these gelling solutions was followed in parallel using a CINAC Continues pH Monitoring System (INRA, France). A water bath was used to maintain a constant temperature of 25°C. The point at which gelation occurred was taken as when the elastic modulus reached a value greater than 1Pa. The corresponding pH at this time was taken as the pH of gel point. The maximum value that the elastic modulus reached within the measurement period was also recorded (G'_{max}).

The turbidity of the solutions as they gelled was measured using a Varian Cary 1 UV/visible spectrophotometer (JVA Analytical, Dublin, Ireland) at a wavelength of 590nm. As the size and/or number of the aggregates increased, more light was scattered leading to an increase in the apparent absorbance of the solutions.

High pressure size exclusion chromatography was used to study the disulphide linked aggregates in the same manner as described above.

2.4 Statistical Analysis

All experiments were carried out in triplicate with the exception of gelation experiments, which were done in duplicate. For the reaction rate kinetics study, each set of experimental data was fitted according to Equation 1 to determine both reaction order and rate. The average values and the standard deviation were calculated. The R^2 value (square of the Pearson product moment correlation coefficient) of how well

the kinetic model fitted the experimental data was determined in Microsoft Excel. 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 was used to determine differences between treatment means. Student's t-tests were performed on the results from the gelation experiments using Microsoft Excel, assuming one-tailed distribution and equal variance for all experimental data sets. Treatment means were considered significantly different at $P \leq 0.05$ unless stated otherwise.

3. RESULTS

3.1 β-lactoglobulin denaturation and aggregation

The reverse phase-HPLC analysis of the protein mixtures of β-lg and BSA that were heated at 78°C for up to 30 min are shown in Fig. 1. Under the conditions used in this study there was little native BSA remaining after 3 minutes. This was in agreement with Hines et al. (1993) who found that BSA denatured at a faster rate than β-lg. The data for the β-lg denaturation were fitted according to Equation 1. This kinetic model gave good curve fits with R^2 values greater than 0.99. The experimental data and the fitted curves as well as the corresponding BSA concentration are shown in Fig 2. The corresponding values for the reaction rate and order using Equation 1 are shown in Table 1.

Insert Fig. 1. & 2. here

Experimental data were also fitted according to a first order reaction kinetics (Equation 2). However, the results were of inferior quality with R^2 values ranging from 0.93 to 0.98, as can be seen in Fig. 3. Therefore, first order kinetics can be ruled

out as a possible mechanism for β-lg denaturation under the experimental conditions used in this study.

Insert Fig 3. & Table 1. here

The reaction order for β-lg in the absence of BSA was found to be 1.4±0.09 under the conditions used in this study. The reaction order for the β-lg denaturation varied with the ratio of β-lg to BSA. Replacement of one tenth of the β–lg by BSA (90:10 ratio) did not change the reaction order significantly ($P<0.05$). However, further replacement of β-lg by BSA to ratios of 80:20 and 75:25 significantly increased the denaturation order to 1.52±0.04 and 1.67±0.13 respectively.

The reaction rate was also dependent on the β-lg : BSA ratio. The differences in the mean values among the treatment groups were greater than would be expected by chance (statistically significant difference $P = 0.001$). Replacing 20% or more β–lg by BSA without changing the total protein content caused a significant increase ($P<0.05$) in the denaturation rate of β–lg, from $2.57\pm0.30 \times10^{-3}(gL^{-1})^{(1-n)}s^{-1}$ (5% β-lg w/w) to $5.07\pm0.72 \times10^{-3}(gL^{-1})^{(1-n)}s^{-1}$ (75 : 25 β-lg : BSA; w/w), which indicated that β-lg was denatured significantly faster in the presence of BSA. In contrast to that, when the β-lg concentration was simply reduced without being replaced by any BSA, i.e. samples were diluted to β-lg concentration equivalent to those above, the denaturation rate decreased significantly ($P<0.05$) from $2.57\pm0.30 \times10^{-3}(gL^{-1})^{(1-n)}s^{-1}$ to $1.92\pm0.07 \times10^{-3}(gL^{-1})^{(1-n)}s^{-1}$ (at 5% and 4% w/w respectively) whereas the reaction order did not change significantly ($P<0.05$). This shows that BSA exerts a major influence on the denaturation of β-lg.

The particle size of the aggregates formed during the heating stage was measured by dynamic light scattering. Results are shown in Table 1. Mean particle

sizes decreased significantly ($P<0.05$) in the presence of BSA. This is despite the fact that BSA is estimated to be two to four times larger than β-lg (~diameter 4nm) (Peters, 1985; He & Carter, 1992; Brownlow et al., 1997). The larger molecular weight of BSA means that the overall molar concentration of protein is lowered by the addition of BSA. One could therefore expect a small decrease in particle size due to a decrease in molar protein concentration (Alting et al., 2003). However, the change in the particle size is only significant between the 100: 0 and the 90:10 β-lg : BSA samples and does not change significantly upon further addition of BSA. However it is worth noting that with aggregates this small, the limit of detection of the instrument becomes a factor and caution must be exercised in the interpretation of the results.

Two-dimensional gel electrophoresis was used to examine if the heat-induced aggregates contained both BSA and β-lg Fig. 4. In the first dimension of the gel electrophoresis, non-reduced, disulphide-linked aggregates were separated by their size. Most of the aggregates remained at the top of the gel. Some monomers and dimers of β–lg could also be detected. When the aggregates were reduced by mercaptoethanol and thereby intra- and intermolecular disulphide bonds cleaved, only two bands were separated in the second dimension on the 15% PAGE gel, namely BSA and β-lg monomers. Both proteins were present in all the larger aggregates, whereas some very small aggregates only contained β-lg. However, this result clearly confirmed similar observations (Gezimati et al., 1996; Havea et al., 2001) that all of the BSA was involved in covalently linked aggregates with β-lg.

Heat induced aggregates that are exclusively linked by disulphide bonds were also separated by **size exclusion chromatography**. The treatment of the sample with the buffer at pH 7.0 containing SDS ensured that the electrostatic and hydrophobic interaction between proteins are negligible. IAA in the buffer blocked any remaining

free sulphydryl groups to prevent further formation of disulphide bonds. This treatment meant that only disulphide linked aggregates remained in the sample. This unfolding of the proteins makes it difficult to determine the molecular weight of the aggregates based on their retention time as the shape is critical to determining the retention time of proteins on GPC columns. The β-lg sample (no added BSA) exhibited a different chromatography profile to that of samples containing BSA (Fig. 5). The sample without BSA contained two types of aggregates (labelled 1 and 2 in Fig. 5) which eluted between the void volume at 7.0mL and monomeric β-lg at 12.5mL. Bands 1 and 2 were not present in chromatograms derived from samples that contain BSA.

Insert Fig. 4 and 5 here

3.2 Acid Gelation

Elastic modulus (G'), turbidity and pH were monitored as a function of time during the acidification and gelation of the denatured protein mixture. Fig. 6 and Fig. 7 show the G', turbidity and pH traces for slow and fast gelation, with 0.15% and 0.5% GDL respectively. Results of gel strength, time and pH of gel point are summarised in Table 2.

Insert Table 2. here

The presence of BSA appeared to decrease the buffering capacity of the solution causing the pH to decrease faster and gelation to occur earlier. This is more apparent in the case of the slow gelation, Fig 7. The pH at which gelation occurred varied between 5.57 and 5.68, but there was no relationship observed between the pH of gelation and the BSA concentration. A gel consisting of only BSA was formed

under the same experimental conditions as for the β-lg/BSA mixtures. The pH rapidly declined and a very weak gel (G'_{max}<80Pa) formed and re-dissolved rapidly (Fig. 6) showing that BSA does not aggregate well itself under these conditions and few disulphide bonds are formed. Previous studies have shown that the rate of denaturation can affect the number of disulphide bonds being formed (Alting et al., 2000). The study showed that, the size of the covalently linked aggregates was inversely related to the concentration of GDL used to acidify the solutions.

The effect of replacing β-lg by BSA in the gels had contrasting effects depending on the rate of acidification of the protein solutions. The gels that were formed rapidly using 0.5% GDL showed a significant ($P<0.05$) decrease in gel strength when BSA was included (Fig. 6). This was the reverse to what has been reported for heat-set gels containing β-lg and BSA (Hines et al., 1993; Matsudomi et al., 1994; Gezimati et al., 1996). When a lower GDL concentration of 0.15% was used, a much slower pH decrease was observed and the gel took longer to form (4 to 6 hours). Again the rate of pH decrease was greater in the presence of BSA and the gels formed at an earlier time. In this case the addition of BSA had the opposite effect compared to that of fast gelation. The gel strength was increased significantly ($P<0.05$) when 25% of β-lg was replaced by BSA. This result was more in line with those of heat-set gels studied previously (Matsudomi et al., 1994; Gezimati et al., 1996).

The turbidity of the gelling solutions increased earlier when BSA was added. This means that aggregates large enough to scatter light were formed more rapidly when BSA was involved. The implication of this is that even though large aggregates were being formed earlier in the process, they did not necessarily yield stronger gels. The apparent absorbance began to increase 14 to 18 minutes before the onset of

gelation in the solutions acidified with 0.5% GDL. At the lower GDL concentration (0.15%) the apparent absorbance increased 18 to 40 minutes before the onset of gelation. During acidification of the heat denatured protein solutions the turbidity of the gels containing only β-lg increased at a slower rate than the solutions containing β-lg/BSA mixtures. In the case of the slowly acidified gels (0.15% GDL) the maximum absorbance reached by the gels was also greater for β-lg/BSA mixed gels. There was a rapid change from transparent gels to opaque gels when BSA was present, showing that BSA also has the ability to alter the transparency of gels.

Insert Fig. 6, 7 & 8 here

Size exclusion chromatography was used again to study the disulphide linked aggregates in the acid gels. For the gelled samples the profile of the β-lg gel was the same as that of the BSA/β-lg gels, with the exception of one extra peak corresponding to a large aggregate eluting at 10.5mL (labelled 3 in Fig. 8 for fast gelation using 0.5% GDL), which was present in the samples containing BSA, it is possible that this peak is due to a small amount of monomeric BSA remaining as BSA has been shown to elute at this retention time. It is important to remember that the samples have been filtered through a 0.45μm filter prior to injection. This means that very large aggregates were removed and not available for analysis by this method. This was particularly relevant in the case of the gelled samples where a large quantity of the protein was removed by the filtering process. This can be seen by comparing the scale of the y axis in Fig. 8 to that of Fig. 5. The peak at 22 mL in Fig. 8 is due to the IAA in the sample. The samples which had been gelled with 0.15% GDL had most of the protein removed by the filtering process (chromatograms not shown). This was an indication that there were larger disulphide linked aggregates formed in the gels which were acidified at a slower rate (0.15% GDL).

Looking at the scales of the chromatograms it is clear that there is more monomeric β-lg in the heated samples (fig 5.) than in the gelled sample (fig 8.). The peaks for the aggregates next to the β-lg are probably made up of β-lg dimers, trimers and tetramers as well as BSA monomers. These aggregates far exceed the intensity of the β-lg peak in the heated sample but in the gelled sample the monomer peak is larger than the peak from the aggregates, this is because there has been more disulphide bond formation and the aggregates are too large for the column.

3. Discussion

The reaction order for the denaturation of 5% (w/w) β-lg in the absence of BSA was found to be 1.4±0.1 under the conditions used in this study. A variety of different reaction orders had been reported previously. Croguennec et al. (2004) reported a reaction order of 2.0, where work was carried out at much lower protein concentrations (0.7%). Verheul, Roef & de Kruif (1998) reported a reaction order of 1.7 under similar conditions and concentrations, whereas Hoffman & Van Mil (1999) reported an order of 1.5 at pH 6.8.

A wide range of values have also been reported for the denaturation rate of β-lg. This study found a rate of $2.57\pm0.3\times10^{-3}(gL^{-1})^{(1-n)}s^{-1}$ for the rate of denaturation of a 5% β-lg solution, under similar pH conditions but much lower protein concentrations (0.7%) rates of $0.35\times10^{-3}\times10^{-3}(gL^{-1})^{(1-n)}s^{-1}$ and $0.85\times10^{-3}(gL^{-1})^{(1-n)}s^{-1}$ have been reported for β-lg A and β-lg B respectively (Croguennec et al., 2004). In skim milk the rate of denaturation was found to be faster; for β-lg A $1.68\pm0.51\times10^{-3}(gL^{-1})^{(1-n)}s^{-1}$ and for β-lg B $2.04\pm0.4\times10^{-3}(gL^{-1})^{(1-n)}s^{-1}$ (Oldfield et al., 2005).

The present study showed that BSA increased the rate of denaturation of β-lg. The rate of denaturation nearly doubled from $2.57\pm0.3\times10^{-3}(gL^{-1})^{(1-n)}s^{-1}$ to

$5.07 \pm 0.72 \times 10^{-3} (gL^{-1})^{(1-n)} s^{-1}$, when β-lg was partially replaced with BSA (75 : 25 ratio; w/w). The effect of BSA on the β-lg denaturation has been studied previously (Hines et al., 1993). There was a 7 fold increase in the denaturisation rate of β-lg from $0.057 \times 10^{-3} (gL^{-1})^{(1-n)} s^{-1}$ to $0.394 \times 10^{-3} (gL^{-1})^{(1-n)} s^{-1}$ when BSA was added. However as discussed in the introduction, the conditions and ratios of protein used in the study were vastly different to the ones used here. Reaction rates were calculated, assuming a reaction order of 2.0.

Here, the question of possible denaturation mechanism arises. A mechanism for β-lg denaturation under conditions similar to those used in this study has been proposed previously (Roefs & de Kruif, 1994). However, the rapid denaturation of BSA during the initial stages of heat denaturation in this study could alter the mechanism of the protein aggregation. The reaction order increased from 1.4 to 1.67 when 25% of the β-lg was replaced with BSA.

There was a significant difference in both rate and order between a 4% β-lg solution and a 5% protein, 80 : 20 β-lg : BSA (w/w), solution ($P<0.05$). Dynamic light scattering and size exclusion chromatography showed that there were differences in the aggregates formed when BSA was present. Two dimensional electrophoresis showed that all of the BSA in involved in disulphide bonds with other proteins and under the conditions used in this study. No BSA monomers were observed in the heat-treated protein mixtures. Size exclusion chromatography of these aggregates also confirmed the involvement of BSA in disulphide linked aggregates.

Gezimati et al.(1996) studied 10% heat-set gels consisting of mixtures of β-lg and BSA. Gelation was induced by heating the protein solutions from 25°C to 90°C at a rate of $1Kmin^{-1}$. They proposed a mechanism whereby more BSA than β-lg unfolded at lower temperatures (≤70°C), therefore aggregates formed at these lower

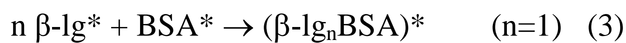
temperatures mainly contained BSA. At higher temperatures, little non-aggregated BSA remained, therefore aggregates consisted primarily of β-lg, in agreement with this our study showed that 60% to 75% of the β-lg was still in its native state by the time when all the BSA had been denatured (Fig 2). This formed the basis for the following aggregation mechanism proposed in this paper.

The experimental data presented in this paper only monitored the disappearance of the native β-lg rather than the appearance of any intermediate or product. However, it can be implied that the reaction order is higher than one and a more complex reaction mechanism is expected. Given that the majority of the solution is β-lg it is possible that a propagation method like that proposed by Roef et al. (1994) could also occur here. Given the similar rates and orders it is conceivable that BSA simply participates in the propagation reaction in the same manner as β-lg.

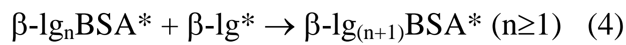
Another possible mechanism for the aggregation is one of "seeding", similar to the process commonly used to start the formation of crystals in solutions. BSA denatures rapidly and is therefore "activated" for further aggregation with itself or other proteins. The denatured BSA could act as "seeds" in the β-lg solution. As the β-lg denatures, it begins to attach to the BSA "seeds", and aggregates can "grow" around a BSA core. The molar ratio of β-lg to BSA decreases with increasing BSA concentrations (90 : 10, 80 :20, 75 : 25 w/w correspond to 33 : 1, 15 : 1, 11 : 1 molar ratio, respectively). The changed molar ratio could explain the increased reaction rate i.e. the presence of more BSA "seeds" could lead to more β-lg being used up in the aggregation reactions. In addition at increasing molar ratios there was also less β-lg to aggregate around each BSA "seed" and aggregates could therefore be expected to get smaller with increasing BSA concentrations. The reaction mechanism could be simplified by the following scheme:

$BSA \rightarrow BSA^*$ (1)

$\beta\text{-lg}_2 \leftrightarrows 2\beta\text{-lg} \rightarrow 2\beta\text{-lg}^*$ (2)



$n\ \beta\text{-lg}^* + BSA^* \rightarrow (\beta\text{-lg}_n BSA)^*$ (n=1) (3)



$\beta\text{-lg}_n BSA^* + \beta\text{-lg}^* \rightarrow \beta\text{-lg}_{(n+1)} BSA^*$ (n≥1) (4)

Reaction (1) proceeds rapidly; the majority of BSA was denatured within the first three minutes heating. The denatured, reactive BSA molecules (BSA*), or "seeds", can then react with a β-lg molecule (reaction 3), which had previously unfolded to become reactive β-lg* (reaction 2). As reaction 3 proceeds, it will push reaction 2 to the right and therefore increasing the overall reaction rate. This mechanism would explain the increased reaction rate. As mentioned earlier the a decrease particle size is only observed between the pure β-lg sample and the samples containing BSA, there is no significant difference between the 90:10, 80:20 and 75:25 samples. However, the size of the latter aggregates are close to the limit of detection of the dynamic light scattering instrument so it would not be prudent to try and fit a kinetic model to these results.

To the authors knowledge this is the first time that it has been reported that BSA could also cause a reduction in gel strength. The decrease in gel strength was observed in gels where the pH was decreasing rapidly (0.5% GDL). As mentioned before, previous studies were always carried out using heat-set gels. Plotting the gel strength against pH (not shown) reveals that the difference in the strength of the pure β-lg gels at the two different GDL concentrations is a result of the difference in the terminal pHs. However in the case of the gels containing mixtures of β-lg and BSA the difference between the two different GDL concentrations is brought about by factors other than the difference in terminal pH. The rate of change of pH has been shown to affect disulphide bond formation in WPI gels. Alting et al. (2000) showed

that increasing the GDL concentration from 0.7% to 1.3% caused the covalently linked aggregates to decrease in size from 277±3 nm to 76±1 nm. The rapidly decreasing pH results in the proteins spending a shorter period of time in the isoelectric pH region (pI equals 5.2 for β-lg and 4.8 for BSA) (Fox & McSweeney, 2003). Around the pI the repulsive forces between the proteins are at their minimum which implies that the proteins can get closer to one another allowing disulphide bonds formation. In this study it was found, that at slower acidification rates (0.15% GDL) BSA enhanced the gel strength compared to that of β-lg gels. This result is in agreement with the work by Matsudomi et al. (1991) where formation of disulphide bonds was found to be more important for the gels containing BSA than for gels containing β-lg.

In order to further confirm the mechanism of the formation of intermolecular disulphide bonds in the pH region around the pI further research needs to be carried out into the gelation of mixed protein systems.

4.0 Acknowledgement

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Legends for Figures.

Fig. 1. Example of a reverse phase HPLC chromatogram. A 90:10 ratio β-lg : BSA at a total protein concentration of 5% (w/w), heated at 78°C for 0, 1, 2, 5, 10, 15, 20 and 30 minutes. C_t/C_0 is the ratio of β-lg concentration at time zero and time t.

Fig. 2. Curve fits of the experimental data for β-lg denaturation at a total protein concentration of 5% (w/w). Points represent experiment data; β-lg : BSA ratios (w/w): 100:0 (diamonds), 90:10 (triangles), 80:20 (squares) and 75:25 (circles). The lines represent the curves fitted to the data using Equation 1; 100:0 (continuous), 90:10 (broken), 80:20 (dotted), 75:25 (broken and dotted). The solid shapes represent the corresponding values for native BSA.

Fig. 3. Example of comparison between first order fit (broken line) and fit using Equation 1 for an 80 : 20 ratio β-lg : BSA at a total protein concentration of 5% (w/w), heated at 78°C.

Fig. 4. Two-dimensional electrophoresis gel of 75:25 β-lg : BSA at a total protein concentration of 5% (w/w), heated at 78°C for 30 min. The first dimension is a 4-15% gradient polyacrylamide gel run under non-reducing conditions; the second dimension is a 15% polyacrylamide gel run under reducing conditions. Stained lane from first dimension is shown as a horizontal insert across top of the second dimension for comparative purposes. The lane on the right hand side of the second dimension gel contains molecular weight standards ranging in weight from 14,400 Da to 66,000 Da.

Fig. 5. HP-SEC profiles for 5% protein solutions heated at 78° for 30 min with and without BSA. Samples have been treated with SDS and IAA so only disulphide linked aggregates remain. The broken line represents sample containing β-lg and BSA in a 75:25 (w/w) ratio. The continuous line represents sample containing only β-lg.

Fig. 6. Effect of BSA on the gelation carried out using a fast rate of acidification, 0.5% GDL, 2% (w/w) total protein. Circles represent the elastic modulus of the gels, squares represent the pH of the gels and the triangles represent the absorbance at 590nm (turbidity) of the gels. The filled shapes represent gels containing only β-lg, the hollow shapes represent gels containing a 75:25 (w/w) mixture of β-lg and BSA, the grey filled shapes represent gels containing only BSA.

Fig. 7. Effect of BSA on the gelation carried out using a slow rate of acidification, 0.15% GDL, 2% (w/w) total protein. Circles represent the elastic modulus of the gels, squares represent the pH of the gels and the triangles represent the absorbance at 590nm (turbidity) of the gels. The filled shapes represent a gel containing only β-lg and the hollow shapes represent a gel containing a 75:25 (w/w) mixture of β-lg and BSA, the grey filled shapes represent a gel containing only BSA.

Fig. 8. HP-SEC profile for soluble covalent linked aggregates extracted from acid gels (2% protein w/w) with and without BSA, 0.5% GDL used. Samples have been treated with SDS and IAA so only disulphide linked aggregates remain. The broken line represents sample containing β-lg and BSA in a 75:25 ratio. The continuous line represents sample containing β-lg.

Captions for tables

Table 1. Summary of results for heat denaturation and aggregation study using 5% (w/w) protein solutions, pH 7.0, heated at 78°C for 30 min.

Table 2. Summary of results for acid gelation of a 2% protein solution (w/w) using a solution which had been previously heated at 5% (w/w) protein concentration, pH 7.0, 78°C for 30 min.

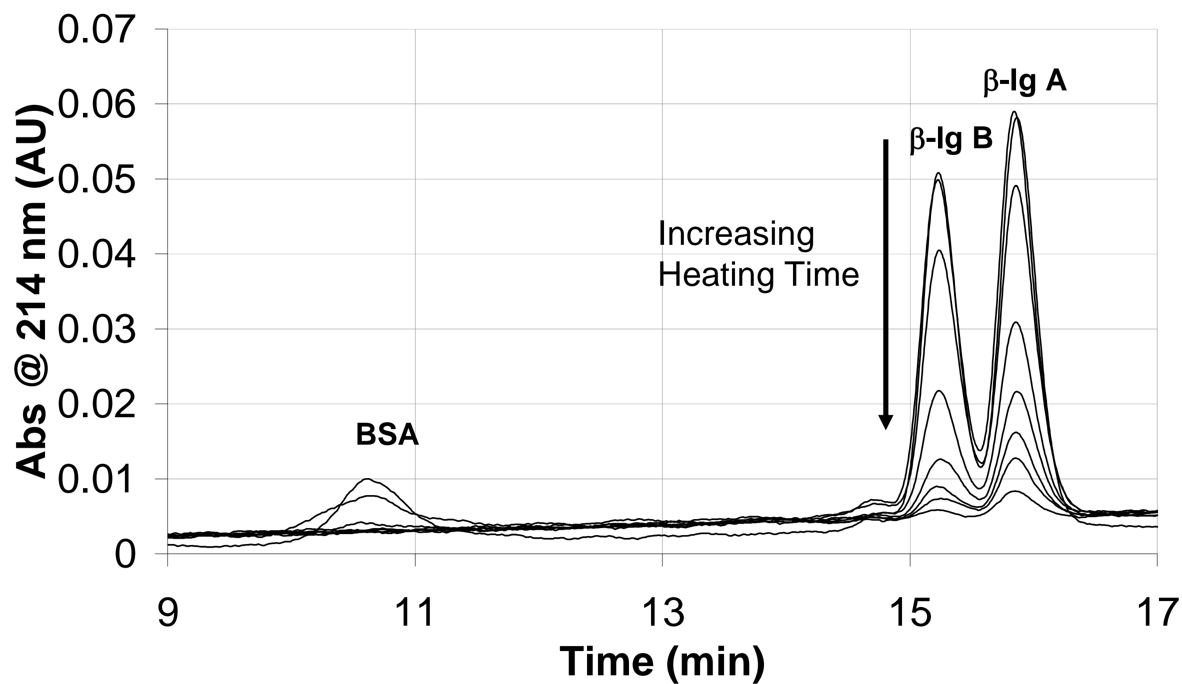


Fig. 1.

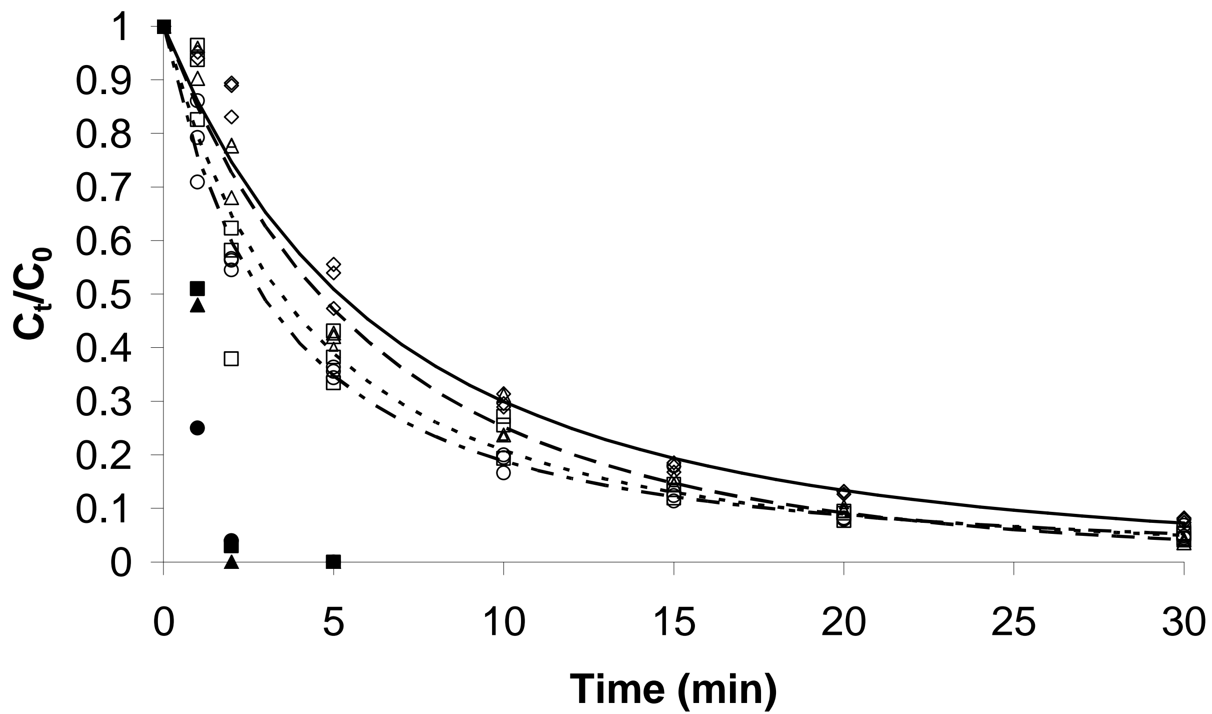


Fig. 2.

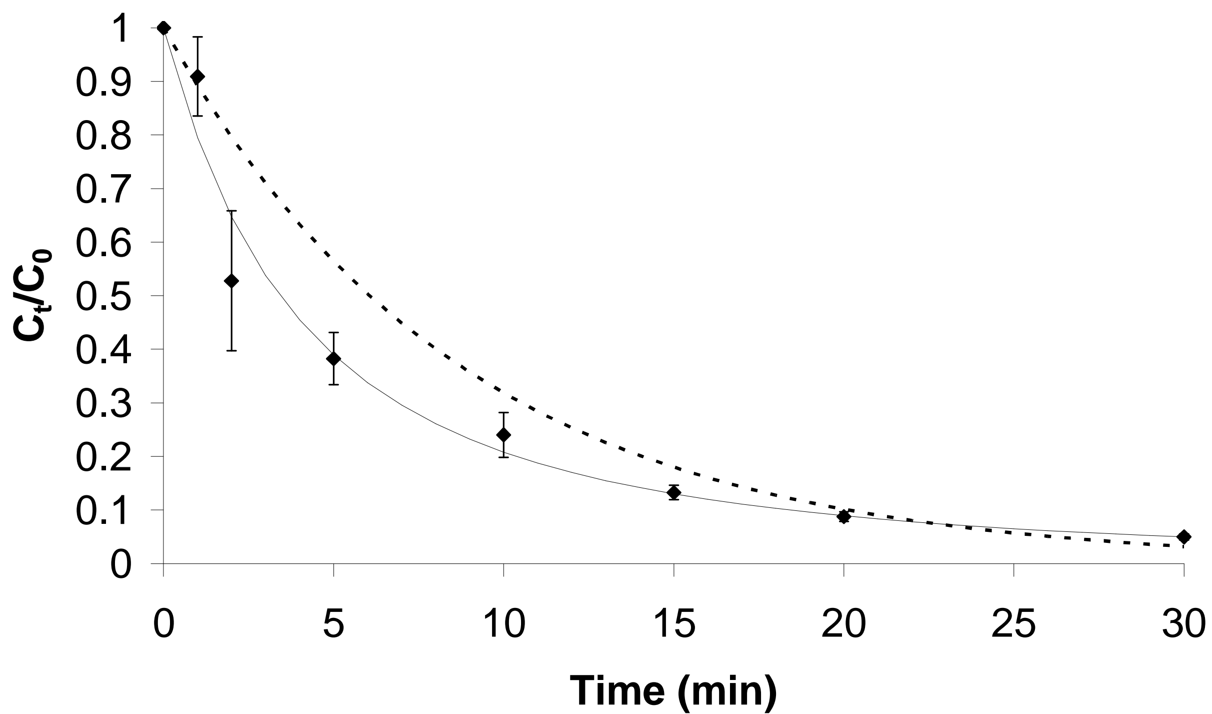


Fig. 3.

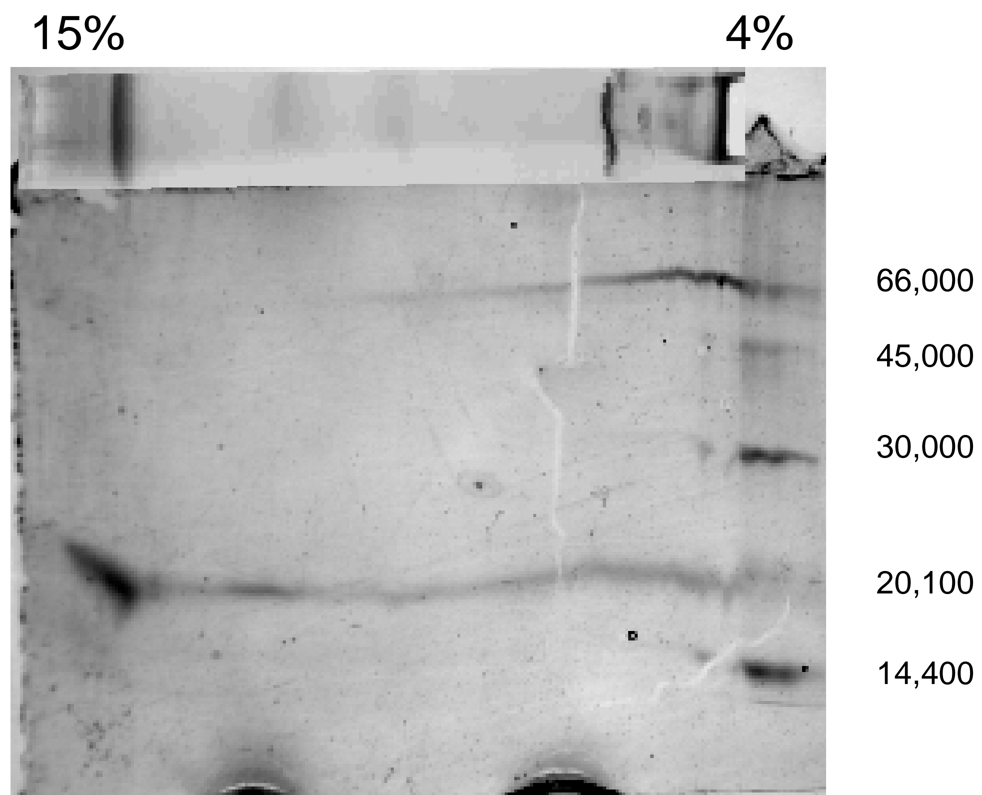


Fig. 4.

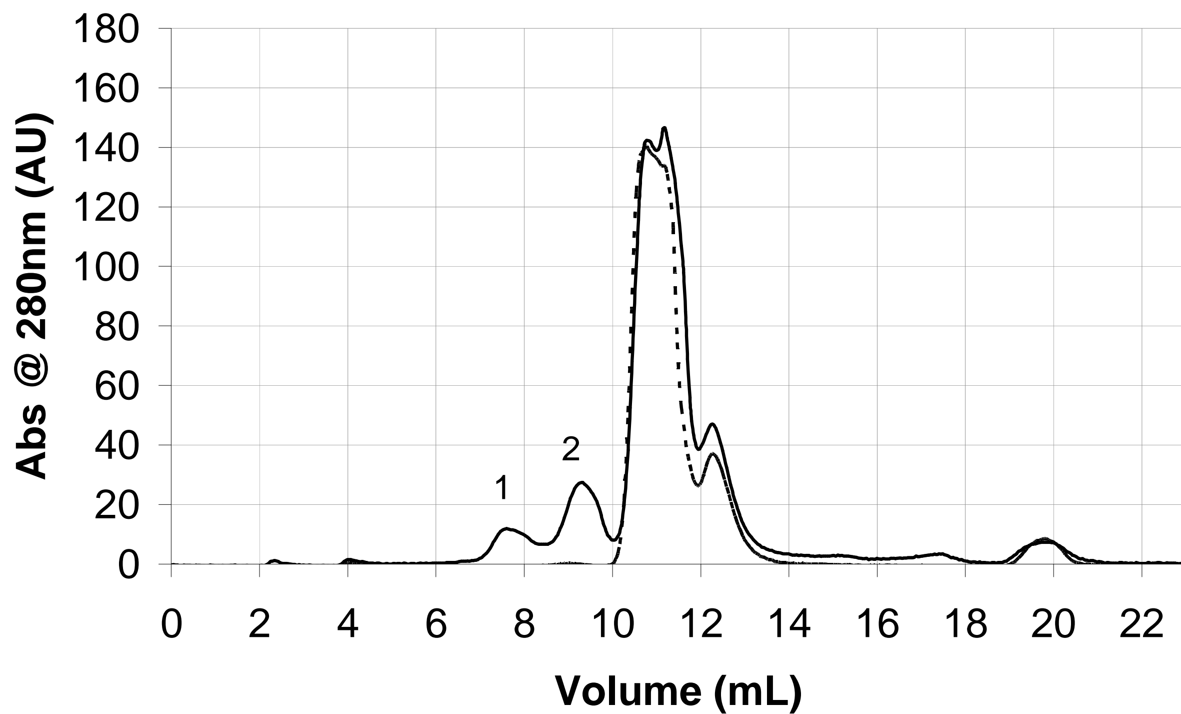
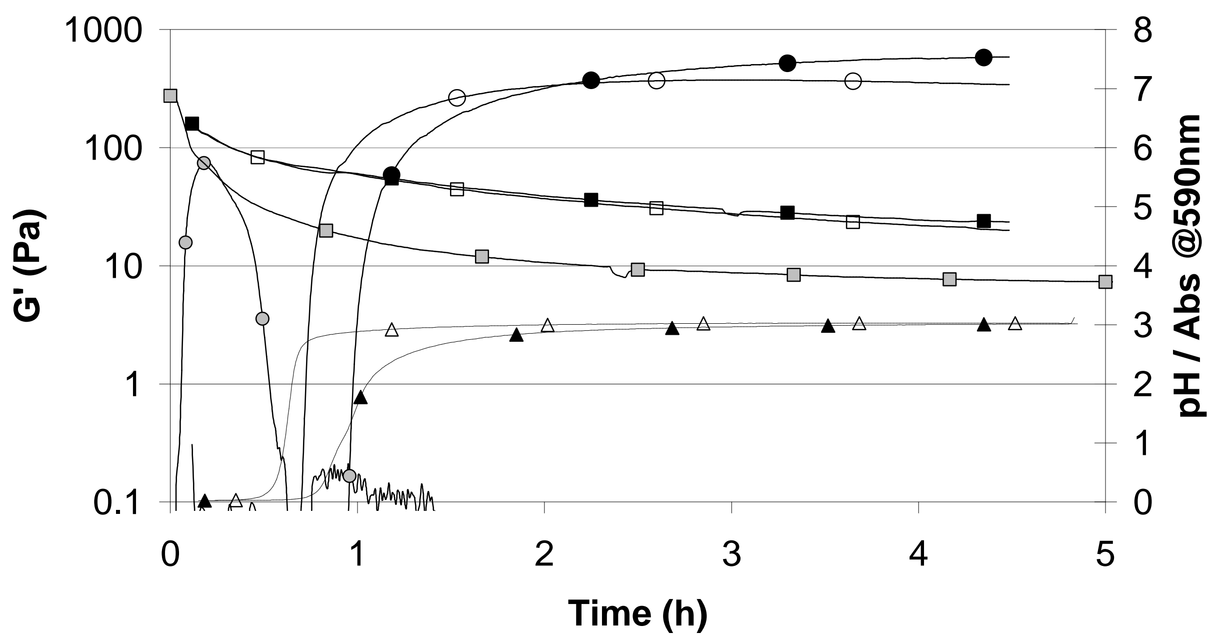


Fig. 5.



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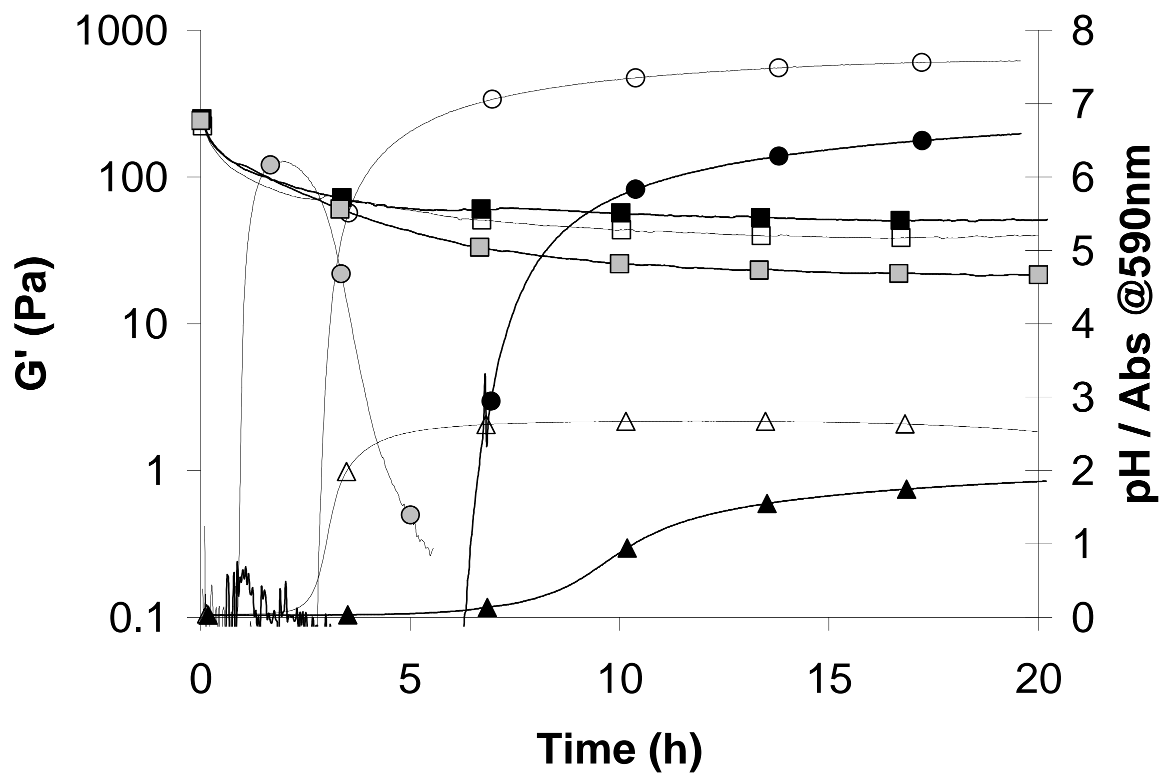

Fig. 7.

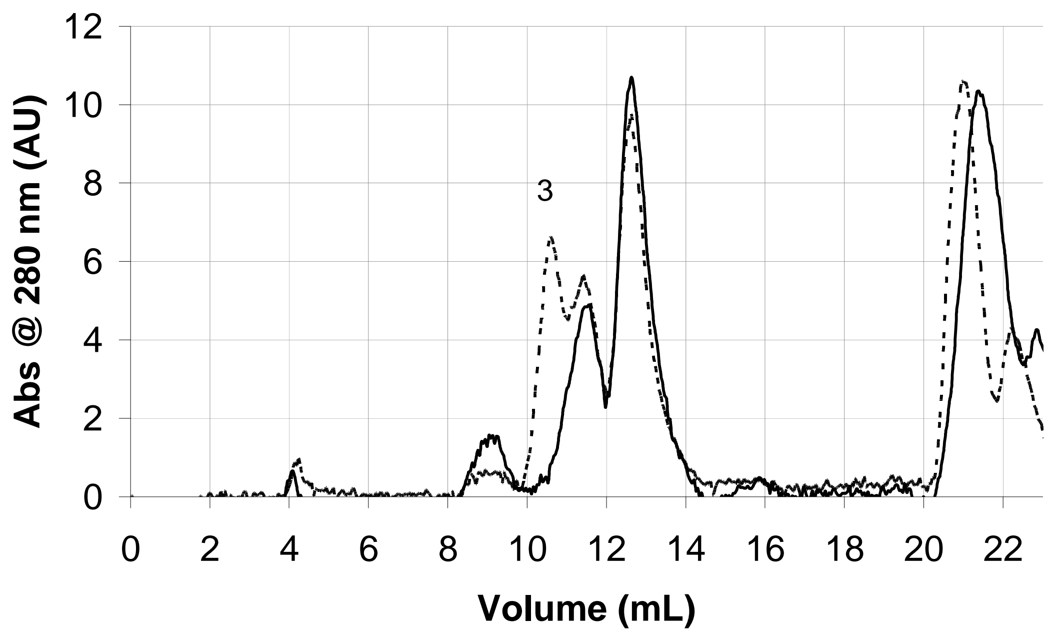

Fig. 8.

β-lg : BSA (w/w)	Aggregate size (nm)	Reaction Order, n	Reaction Rate, k $(gL^{-1})^{(1-n)}s^{-1} \times 10^{-3}$
100 : 0	60±3	1.4±0.1	2.57±0.30
90 : 10	33±7	1.2±0.1	2.73±0.40
80 : 20	27±5	1.5±0.0	3.98±0.55
75 : 25	30±7	1.7±0.1	5.07±0.72

Table 1.

β-lg : BSA (w/w)	% GDL (w/w)	G'_{max} (Pa)	Time of gel point (min)	pH of gel point
100:0	0.5	586±45	62±4	5.55±0.02
90:10	0.5	495±15	50±0	5.65±0.07
80:20	0.5	386±7	46±3	5.61±0.04
75:25	0.5	374±3	44±0	5.68±0.15
100:0	0.15	198	394	5.56
75:25	0.15	621	174	5.71

Table 2.