Research Journal of Dairy Sciences 1 (1-4): 18-23, 2007
ISSN: 1993-5277

Heat-Induced Denaturation/Aggregation of β-Lactoglobulin as Affected by *N*-Ethylmaleimide, NaCl, $CaCl_2$ and pH

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Abstract: The degree of denaturation/unfolding and subsequent aggregation dictate the functional attributes of β-lactoglobulin (β-lg), such as increased water-binding, gelation or general structure/texture development. The heat-induced denaturation/aggregation behaviour of β-lg was examined in the presence of water, NaCl, $CaCl_2$ and/or *N*-ethylmaleimide in the pH range 5.0-7.0. It was shown that rates of unfolding and aggregation of β-lg, were pH dependent, with aggregation being rate limiting at pH 7.0 and unfolding rate limiting at pH 5.0. Results suggested that heat-induced β-lg aggregation at pH 5 under low ionic strength conditions did not initially involve thiol-disulphide interchange reactions. However, secondary formation of thiol-disulphide interchange linkages was time dependent. The addition of NaCl to β-lg solutions prior to heating inhibited denaturation/aggregation on heating at pH 5.0-6.0 but promoted these reactions at pH 6.0-7.0. Over a certain pH range, the presence of *N*-ethylmaleimide promoted the interaction between unfolded β-lg molecules by non-specific forces. The addition of *N*-ethylmaleimide to β-lg solutions prior to heating in the presence of $CaCl_2$, resulted in similar denaturation/aggregation levels at pH values between 5.0 and 5.75 but slightly reduced levels at pH values between 6.0 and 7.0. Results showed the significance of non-specific forces in driving the heat-induced aggregation of β-lg over a range of pH values, which has relevance to acidified food systems.

Key words: β-lactoglobulin, denaturation/aggregation kinetics, NEM, NaCl, $CaCl_2$, pH

INTRODUCTION

The milk protein, β-lactoglobulin is a globular milk protein with a molar mass of 18.3 kDa. Factors influencing the heat-induced aggregation and gelation of β-lg include non-covalent interactions such as ionic, van der Waals and hydrophobic interactions as well as thiol/disulphide exchange reactions, leading to the formation of disulphide-linked aggregates (Mulvihill *et al*., 1990; Hoffmann and Mil, 1997). Whether these aggregation reactions are irreversible or potentially reversible will depend on the degree of chemical or physical aggregation involved (Verheul *et al*., 1998) and on factors such as temperature, pH and ionic strength (Hoffmann *et al*., 1996). The extent of aggregation can extend from the formation of dimers, trimers, oligomers, soluble aggregates etc to large aggregate formation and visible precipitation or gelation (Croguennec *et al*., 2004).

To date most research has been carried out on the denaturation and aggregation of β-lg at neutral pH (Hoffmann *et al*., 1996; Mounsey and Kennedy, 2007) but reactions at acidic pH have not had as much attention. Hoffmann *et al*. (1996) observed that with heating β-lg at pH 6.9, small aggregates were formed by thiol-disulphide interchange reactions. In contrast, very large aggregates were formed at a pH≤6.4, which was attributed to secondary aggregation by non-covalent cross-linking of smaller aggregates formed by thiol disulphide-interchange reactions. O'Kennedy *et al*. (2006) found that on heating β-lg at pH values between 5.0 and 7.0, under low ionic strength conditions, maximum denaturation occurred with heating at pH 5.0, while denaturation was at a minimum at pH 6.0-6.5. Mounsey and Kennedy (2007) concluded that in the presence of stoichiometrically sufficient levels of $CaCl_2$, thiol-disulphide interchange reactions were not required for aggregation and precipitation of β-lg heated at pH 7.0. Whey protein secondary aggregation is a necessary prerequisite to functionality development in the formation of cold-gelling food systems. The aim of this study, was to compare the denaturation/aggregation kinetics of β-lg at different pH values and in different ionic environments and use the information for future work to elucidate the acid gel behaviour of β-lg containing milk protein systems.

MATERIALS AND METHODS

A powder enriched in β-lg (98.2% â-lg and 1.8% α-lactalbumin) was prepared in-house (Moorepark Food

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Research Centre, Moorepark, Fermoy, Co. Cork, Ireland) according to the method of Mounsey *et al.* (2007). *N*-Ethylmaleimide (NEM) and Calcium Chloride ($CaCl_2$) were obtained from Sigma Chemical Co. (St. Louis, MO. USA). All other chemicals were analytical reagent grade and supplied by BDH (Poole, England).

Sample preparations: β-Lg dispersions at a range of concentrations up to 6% (w/w) protein were prepared by dissolving β-lg enriched powder in distilled/deionised water, 100 mM NaCl or 10 mM $CaCl_2$ and stirring at room temperature. Some β-lg samples were dispersed in 5 mM NEM. The pH of the dispersions was adjusted using 1 N NaOH or 1 N HCl.

Kinetics of heat-induced denaturation/aggregation of β-Lg: The β-lg dispersions were heated at various protein concentrations in a series of tightly capped 1.5 mL glass vials containing 1 mL of sample at various pH values in a water bath at 78°C. After 10 min, vials were removed and immediately cooled. Following the heating/cooling step, 0.5 mL of acetic acid/sodium acetate buffer (0.5 N, pH 4.7) was added to 0.5 ml of sample. Samples were transferred into 1.5 mL plastic Eppendorf containers (Sigma, Dublin, Ireland). These samples were then centrifuged at 14,000 g for 30 min in an Eppendorf centrifuge 5417C (Unitech, Dublin, Ireland). The absorbance of the supernatant (appropriately diluted) was determined at 280 nm. Hellma® quartz cuvettes (Hellma GmbH and Co., Mulheim, Germany) were used in a Hitachi U-1100 spectrophotometer (Hitachi Ltd, Tokyo, Japan).

The rate of β-lg denaturation/aggregation was described by an integrated form of the general rate equation (Kessler and Beyer, 1991):

$$\text{For } n \neq 1.0: (C_t/C_0)^{1-n} = 1 + (n-1)\,kt \quad (1)$$

with $k = K_n C_0^{n-1}$

Where C_0 = Initial concentration (g. L^{-1}); C_t = Concentration at a time t (g. L^{-1}); k = Apparent rate constant (s^{-1}); t = Heating time (s); K_n = Rate constant (independent of concentration) for the reaction order n [(g. $L^{-1})^{1-n}$ s^{-1}].

$$\text{For } n = 1.0: \ln (C_t/C_0) = -k\,t \quad (2)$$

The rate constants were obtained with Eq. (1) or (2) using linear regression.

Determination of β-lg using reversed-phase high performance liquid chromatography (RP-HPLC): Some β-lg samples were heat-treated at pH 5.0 before re-neutralisation to pH 7.0. The level of monomeric β-lg was determined using RP-HPLC according to the method of Croguennec *et al.* (2004). Reversed-phase separation was performed on a Vydac C_4 214 TP5415 (150×4.6 cm i.d.) column (Phenomenex, Cheshire, England) connected to the Waters chromatography system. HPLC-grade acetonitrile (Sigma, Dublin, Ireland) containing 0.1% trifluoroacetic acid was used as the eluant under linear gradient elution from 36-48.6% in 15 min and from 48.6-90% in 1 min, at a flow rate of 1 mL min^{-1}. UV absorption was measured at 214 nm. Commercial β-lg (Sigma, Dublin, Ireland) was used as a standard. Samples and the standard were prefiltered through 0.22 μm membrane filters prior to application to the column.

Statistical analysis: The preparation of all solutions and subsequent analyses on them were performed in triplicate. Analysis of Variance (ANOVA) was carried out using SigmaStat (version 3.0; Jandel Scientific, Corte Madera, CA, USA). Tukeys multiple-comparison test was used to determine differences between treatment means. Treatment means were considered significantly different at $p \leq 0.05$.

RESULTS AND DISCUSSION

Effect of NEM on heat-induced denaturation/aggregation kinetics of β-lg in water: The concentration of heat-induced (78°C, 10 min) denatured/aggregated protein in β-lg (1 or 6%, ww^{-1}) in water or 5 mM NEM, at pH values from 5.0-7.0 is shown in Fig. 1. On heating β-lg (1%, ww^{-1}), in the presence of NEM at pH 7.0, less protein denaturation/aggregation was observed compared to heating in water. However, on reduction of the pH of heating, denaturation/aggregation of the protein was significantly ($p \leq 0.05$) increased to a maximum of 76% at pH 5.0. There was no significant ($p \leq 0.05$) difference in denaturation/aggregation levels between heating in water or NEM at pH 5.0 or 7.0. However, at intermediate pH values, protein denaturation/aggregation was significantly ($p \leq 0.05$) increased in the presence of NEM. Sawyer (1968), Xiong *et al.* (1993), Hoffmann and Mil (1997) also observed that non-covalently linked aggregates were formed when β-lactoglobulin was heated in the presence of NEM. The binding of NEM to the free thiol group may increase molecular flexibility and therefore, enhance protein-protein interactions by non-specific bonding (Xiong *et al.*, 1993). This raises the query as outlined by Hoffmann and Mil (1997) regarding the relevance of non-covalently linked aggregate formation when heating in the absence of NEM.

The formation of denatured protein aggregates by non-covalent interactions and/or disulphide interchange

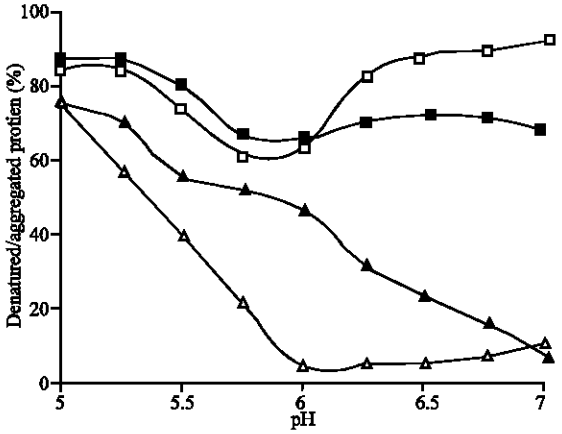

Fig. 1: Percentage denatured/aggregated protein as a function of heating pH (at 78°C for 10 min) for 1% (w/w) β-lg in water (Δ) or 5 mM NEM (▲) or for 6% (w/w) β-lg in water (□) or 5 mM NEM (■). Each curve represents the mean of triplicate trials

reactions may occur simultaneously or sequentially (Hoffman and Mil, 1997). According to the denaturation/ aggregation reaction scheme of β-lg, the denaturation step, consisting of successive unimolecular reactions, is usually considered to be a first-order reaction, while aggregation steps are bimolecular and second-order reactions. Then, the overall reaction order for the disappearance of native β-lg is expected to be between 1 and 2 depending upon the ratio of the reaction rates of the different steps (Verheul *et al*., 1998). However, the decrease in the native protein concentration tends towards order 1 kinetics at lower pH values (5.0) and order 2 kinetics at higher pH values (>7.0), suggesting that the sequential order of non-covalent or disulphide interchange reactions should be pH dependent.

When β-lg was heated at a higher protein concentration (6%, ww^{-1}), in the presence of 5 mM NEM, similar levels of protein denaturation/aggregation were observed on heating at pH values between 5.0 and 6.25, compared to heating in water. However, heating at higher pH values (6.5-7.0), in the presence of NEM, significantly ($p \leq 0.05$) reduced the protein denaturation/aggregation compared to heating in water. It was obvious from Fig. 1 that the β-lg denaturation/aggregation kinetics were dependent on the concentration of protein present during the heating step. At low protein concentrations (1%, w/w), the aggregation step was rate-limiting between pH 6.0-7.0. When the initial reaction rate was plotted as a function of the native protein concentration on a double logarithmic scale, the slope of the line estimates the reaction order (n). The change in the reaction order (n) with pH of heating is shown in Table 1.

The overall reaction was shown to obey second order kinetics when heated in water or NEM at pH 7.0, which

Table 1: Reaction order (*n*) of β-lg denaturation/aggregation following heating at 78°C for 10 min in water or 5 mM NEM. Each value represents the mean of triplicate trials

pH	Water	NEM
7	2.23	2.2
6.75	2.36	1.82
6.5	2.25	1.62
6.25	2.42	1.44
6	2.38	1.19
5.75	1.58	1.14
5.5	1.34	1.13
5.25	1.23	1.12
5	1.05	1.08

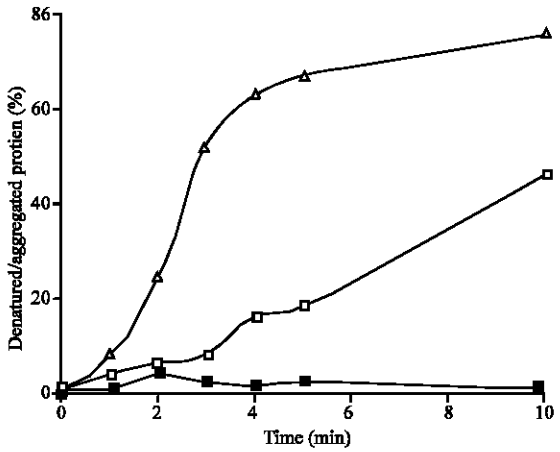

Fig. 2: Percentage aggregated protein as a function of heating time for 1% (w/w) β-lg, heated at 78°C, pH 5.0 (Δ); 1% (w/w) β-lg, heated at 78°C, pH 5.0, cooled and reneutralised to pH 7.0 in water (□) or in 1.67 mM NEM (■). Each curve represents the mean of triplicate trials

made the aggregation step, rate limiting. Hoffmann and Mil (1997, 1999) concluded that with heating β-lg at pH 7.0, aggregation was mainly as a result of thiol-disulphide interchange chemical reactions and at low protein concentrations these chemical reactions were particularly limiting. The reaction order for β-lg was relatively unchanged with heating in water at pH values from 7.0 to 6.0. However, incremental reduction of the heating pH to 5.0 resulted in an incremental reduction of the reaction order to 1.0. Heating in the presence of NEM promoted aggregation of denatured β-lg molecules through non-specific interactions, especially at low protein concentration, thus decreasing the order of the overall reaction, especially at higher pH values.

Formation of physical bonds between proteins is enhanced at pH values close to the isoelectric point because of decreased intermolecular repulsion and decreased solubility of the protein (Verheul *et al*., 1998). In addition the reactivity of the free thiol group is reduced at acidic pH values (Donovan and Mulvihill, 1987). This would suggest that aggregate formation on heating at pH

5.0 is, at least initially, dependent on physical or non-specific bonds. Figure 2 shows the fractional concentration of non-aggregated protein as a function of heating time of 1% (ww^{-1}) β-lg in water or NEM, which had been heated at pH 5.0 and 78°C before immediate re-neutralisation to pH 7.0. Subsequent analysis using reverse-phase HPLC suggested that protein aggregates were largely reversible to the monomer for a limited time of heating (5 min). Heating for longer times induced a degree of irreversibility as indicated by reduced levels of soluble protein. If thiol-disulphide interchange reactions, which may have occurred during the heating or cooling steps, were inhibited in the presence of NEM, then reversibility of aggregation should have proceeded when electrostatic/ hydrophobic effects were re-balanced by pH adjustment. No change in the profiles was observed in the presence of NEM on heating, which indicated that the physical or non-specific bonds formed on heating at pH 5.0 were reversible on re-neutralisation. This is in agreement with Otte *et al.* (2000) who observed that a 5% (ww^{-1}) β-lg gel formed through heating at pH 5.0 could be solubilised in SDS without a reducing agent. They concluded that the gel network formed by heating at pH 5.0 was more dependent on non-covalent bonds or lower concentrations of β-lg, than at higher protein concentrations.

It is suggested that exposure of the free thiol group and subsequent thiol-disulphide interchange reactions were not necessarily a precondition for protein aggregation reactions during heating at pH 5.0. Aggregation of protein during heating at the isoelectric point may require minimal perturbation of the native protein configuration to initiate the secondary aggregation reactions. However, the possibility of delayed formation of disulphide bonds initiated by the protein concentration effect of the aggregation process is always likely, especially at high temperatures. While the presence of NEM did not affect the kinetics of aggregation during the heating step, it does inhibit any delayed thiol-disulphide interchange reactions, which may subsequently occur, either during the heating procedure or on cooling. This substantiates the suggestion that physical or non-covalent forces are mainly responsible for the initial β-lg aggregation observed on heating at the isoelectric point.

Secondary formation of covalent bonds (thiol-disulphide interchange reactions) probably contribute to the integrity and irreversibility of the three-dimensional matrix. Alting *et al.* (2000) have shown that covalent disulphide bond formation can occur under acidified conditions once a protein network was formed. The delayed formation of disulphide bonds was also observed for high-pressure induced β-lg gels (Dumay *et al.*, 1998) and acid milk gels (Vasbinder *et al.*, 2003). While thiol-disulphide interchange reactions were a significant factor during heat-induced β-lg aggregate formation at alkaline pH (7.0-8.0) and significantly contributed to their irreversibility (Hoffman and Mil, 1997), lowering the pH to 5.0 significantly slowed these reactions (Bryant and Mc Clements, 1998).

Effect of NEM on denaturation/aggregation kinetics of β-lg in 100 mM NaCl: The concentration of heat-induced (78°C, 10 min) denatured/aggregated protein in β-lg (1 or 6%, ww^{-1}) in 100 mM NaCl ± 5 mM NEM, at pH values from 5.0 to 7.0 is shown in Fig. 3. Increasing the ionic strength through addition of NaCl enhances denaturation/ aggregation on heating at neutral pH and inhibits at acidic pH values. This was particularly obvious where 6% (ww^{-1}) protein was heated in the presence of salt where the samples gelled after 10 min of heating at 78°C. When β-lg in 100 mM NaCl was heated at 1 or 6% (ww^{-1}) levels in NEM, the levels of denaturation/aggregation were reduced at the pH values of 6.5-7.0, but increased at lower pH values of 5.0-6.25. The binding of NEM to the free thiol group, even in the presence of NaCl, may increase molecular flexibility and therefore, enhance protein-protein interactions by non-specific bonding (Xiong *et al.*, 1993). Increased levels of denaturation/ aggregation were obtained at all pH values with heating at the higher protein level.

The combined effect of a reduced denaturation rate and an increased aggregation rate led to a minimum in β-lg denaturation at pH 6.0 in the presence of NaCl. The reaction order for β-lg heated in 100 mM NaCl increased from 1.52 at pH 5.0-2.21 at pH 5.75 and decreased at higher pH values to 1.15 at pH 7.0. Croguennec *et al.* (2004) also found that the reaction order of β-lg decreased (n = 1.11-1.35) on heating at 85°C and pH 6.5 in 15 mM NaCl compared to in water (n = 2). In addition, Verheul *et al.* (1998) found lower n values of 1.0 and 1.4 for β-Lg heated at 68.5°C in 500 mM NaCl at pH 6.5 and 7.0, respectively compared to values of 1.5 and 1.7 with heating in water. They concluded that the decrease in n was caused by an increase in stability of β-lg in the presence of NaCl, which decreased the rate of the first-order unfolding reaction (denaturation) (Verheul *et al.*, 1998). The second order reaction kinetics with heating in NaCl at the lower pH values of ~ 5.0 -6.25, made the aggregation step, rate limiting. The reaction order for β-lg heated in 100 mM NaCl and 5 mM NEM increased from 1.2 at pH 5.0 to 1.61 at pH 7.0. The reaction order was higher in the presence of NEM at pH values of 6.5 to 7.0 but lower at pH values between 5.0 and 6.25.

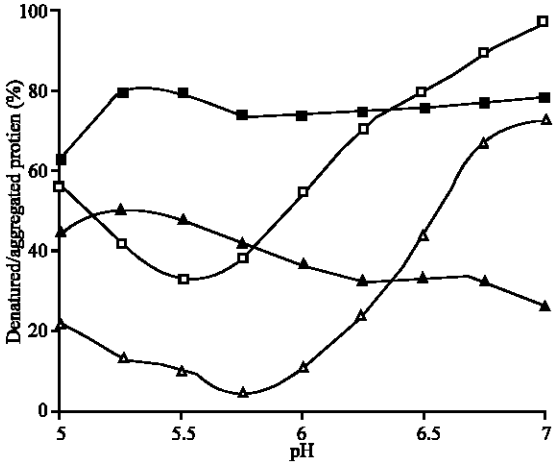

Fig. 3: Percentage denatured/aggregated protein as a function of heating pH (78°C, 10 min) for 1% (w/w) β-lg in 100 mM NaCl (Δ) or 100 mM NaCl and 5 mM NEM (▲); 6% (w/w) β-lg in 100 mM NaCl (□) or 100 mM NaCl and 5 mM NEM (■). Each curve represents the mean of triplicate trials

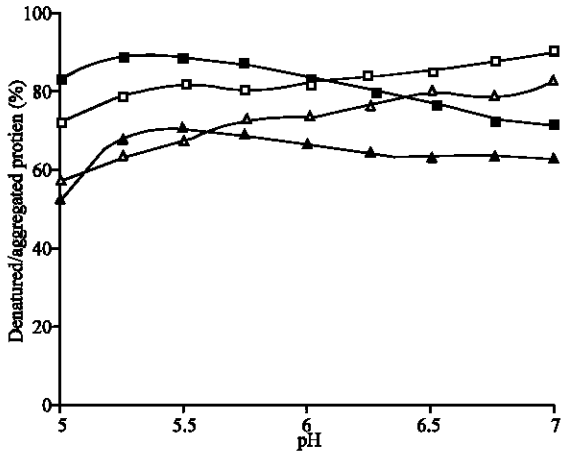

Fig. 4: Percentage denatured/aggregated protein as a function of heating pH (78°C, 10 min) for 1% (w/w) β-lg in 10 mM $CaCl_2$ (Δ) or 10 mM $CaCl_2$ and 5 mM NEM (▲); 6% (w/w) β-lg in 10 mM $CaCl_2$ (□) or 10 mM $CaCl_2$ and 5 mM NEM (■). Each curve represents the mean of triplicate trials

Denaturation/aggregation kinetics of β-lg in 10 mM $CaCl_2$: The concentration of non-aggregated protein in 1 or 6% (w/w) β-lg in 10 mM $CaCl_2$ or 10 mM $CaCl_2$ and 5 mM NEM, which had been heated for 10 min at 78°C was plotted against heating pH (Fig. 4). Simons *et al.* (2002) suggested that calcium bound to β-lg carboxylate groups with a threshold affinity. Subsequent site-specific screening of surface charges resulted in protein aggregation, driven by the partial unfolding of β-lg at elevated temperatures, which was facilitated by the absence of electrostatic repulsion. Sherwin and Foegeding (1997) demonstrated that aggregation rates were affected by $CaCl_2$/protein stoichiometry rather than the $CaCl_2$ and protein concentrations separately. When 1 % (ww^{-1}) β-lg in 10 mM $CaCl_2$ was heated in the presence of NEM, the level of denaturation/aggregation were unchanged at pH values between 5.0 and 5.75 but slightly reduced at pH values between 6.0 and 7.0. When 6% (ww^{-1}) β-lg in 10 mM $CaCl_2$ was heated in the presence of NEM, the level of denaturation/aggregation were increased at pH values between 5.0 and 5.75 but reduced at pH values between 6.0 and 7.0. Mounsey and Kennedy (2007) concluded that in the presence of stoichiometrically sufficient levels of $CaCl_2$, thiol-disulphide interchange reactions were not required for aggregation and precipitation of β-lg heated at pH 7.0. The presence of 3 mM $CaCl_2$ on heating, reduced the zeta potential of β-lg from -18.7±1.2-5.2±0.5 mV, which resulted in heat-induced aggregation and gross precipitation (Mounsey and Kennedy, 2007). The level of denatured/aggregated protein is therefore an interactive effect between pH reduction and calcium binding.

CONCLUSION

In conclusion, on heating β-lg at 78°C, it was shown that rates of unfolding and aggregation were pH dependent, with aggregation being rate limiting at pH 7.0 and unfolding rate limiting at pH 5.0. Thiol-disulphide interchange reactions were shown to be necessary for protein-protein aggregation reactions to occur on heating β-lg at pH 7 under low ionic strength conditions. However, protein aggregation reactions occurred readily in the absence of thiol-disulphide interchange at pH 5.0 and under low ionic strength conditions. Secondary formation of thiol-disulphide interchange linkages was time dependent on heating β-lg at pH 5.0. The addition of NaCl to β-lg solutions prior to heating inhibited denaturation/aggregation on heating at pH 5.0-6.0 but promoted these reactions at pH 6.0-7.0. The addition of *N*-ethylmaleimide to β-lg solutions prior to heating in the presence of $CaCl_2$, resulted in similar denaturation/aggregation levels at pH values between 5.0 and 5.75 but slightly reduced levels at pH values between 6.0 and 7.0. Results showed the significance of non-specific forces in driving the heat-induced aggregation of β-lg over a range of pH values, which has relevance to acidified food systems.

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