The Effect of Molecular Chaperone, Alpha-Crystallin, on the Heat-Induced Aggregation of Beta-lactoglobulin

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Abstract: Aggregation of beta-lactoglobulin occurs mainly via intermolecular disulphide bond exchange. Upon heating, beta-lactoglobulin aggregated which increased with increasing pH. The presence of DTT led to more rapid aggregation and precipitation of beta-lactoglobulin. Alpha-Crystallin prevented the aggregation of heat-stressed beta-lactoglobulin and was a more efficient chaperone at higher pH values. In the presence of DTT, however, alpha-crystallin was a less efficient chaperone due to faster aggregation of heated and reduced beta-lactoglobulin.

Key words: Beta-lactoglobulin, alpha-crystallin, crowding agent

INTRODUCTION

Beta-lactoglobulin is a major whey protein of milk[1-3], which at physiological pH values exists as a dimer[4,5]. Each monomer has a molecular weight of 18.3 kDa, contains 162 amino acid residues, two disulphide bonds (C106-C119 and C66-C160) and one free cysteine residue (C121)[6-8]. Upon heating, beta-lactoglobulin dissociates into monomers, leading to exposure of hydrophobic groups and the free sulfhydryl group[9,10]. This then gives rise to aggregation and/or polymerization[4,5,9,11,12]. Beta-lactoglobulin is sensitive to heating at 60-100°C. During heating, the protein undergoes an initial dissociation of dimers followed by conformational changes which increase the exposure of previously buried hydrophobic groups and the thiol group[4,9,12,13,14].

Alpha-Crystallin is the major protein component of the mammalian eye lens, making up 50% of its dry weight[15,16]. It belongs to the sHsp family[17] and acts as a molecular chaperone[17,18] by preventing aggregation of target proteins (e.g. beta and gama-crystallins) under stress conditions through the formation of stable, soluble high-molecular mass complexes[19,20].

Aggregation of beta-lactoglobulin occurs mainly via intermolecular disulphide bond exchange, i.e. the aggregation is not (in the main) due to nucleation-dependent processes[7,9] but arises from polymerization. In a previous study[21] indicated that alpha-crystallin did not inhibit the aggregation of a serpin (AT (antitrypsin)) which aggregated via a polymerization reaction but did inhibit a serpin aggregation (ACT (antichymotrypsin)) when occurring via a nucleation-dependent mechanism.

Therefore, in order to explore the effect of alpha-crystallin on beta-lactoglobulin aggregation, a heating assay of beta-lactoglobulin at different pH values in the presence and absence of alpha-crystallin was performed.

MATERIALS AND METHODS

Chemicals and reagents: Alpha-Crystalline were prepared from bovine lenses obtained from a local abattoir. dithiothreitol (DTT), NaN_3, Acrylamide, Glycine and beta-lactoglobulin were obtained from Sigma-Aldrich (St. Louis, U.S.A.).

Isolation and purification of alpha-, beta- and gama-crystallins from bovine eye lenses: The purification of alpha-crystallin was performed according to the method of Slingsby and Bateman[22] using a Sephacryl S 300HR column with a diameter of 2.6 cm and length of 100 cm in 50 mM Tris, 0.04% NaN_3, pH 7.5 buffer and a flow rate of 20 mL h^{-1}. The elution time of alpha-crystallin was approximately 8-10 h. The purity of the alpha-crystallin fractions was checked by SDS-PAGE (not shown).

Heat stress assay: Beta-lactoglobulin (at a final concentration of 5 mg mL^{-1}) in 50 mM sodium phosphate, 100 mM NaCl and 2.5 mM EDTA, 0.05% (w/v) NaN_3 was incubated at 70°C at different pH values (6.5, 7.0, 7.5 and 8.0) in the presence and absence of 1:1 w:w ratio of beta-lactoglobulin: alpha-crystallin and/or in the absence and presence of DTT. Samples were prepared in 1 cm quartz cuvettes to a final volume of 1 mL. To avoid evaporation from cuvettes, a Seal Plate strip was used made of plastic stable up to 120°C.

Aggregation of beta-lactoglobulin was monitored as light scattering at 360 nm over 3 h with data collected every 2 minutes using a Cary 500 Scan UV-VIS- NIR spectrophotometer.

Protein characterization by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE): Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used to assess the extent of aggregation of heated beta-lactoglobulin in the presence and absence of alpha-crystallin at different pH values.

About 100 μL of heated beta-lactoglobulin solution was taken and pellets were removed by centrifugation. Proteins were dissolved in 25 μL of phosphate buffer and 25 μL of non-reduced loading dye buffer. To obtain information on heated beta-lactoglobulin, which was not aggregated, 25 μL of supernatant of heated sample were taken and then 25 μL of reduced buffer were added to supernatant sample.

Samples were boiled and then loaded on to 15% (v/v) polyacrylamide resolving and 4% (v/v) stacking gel for separation. A Bio-Rad MiniProtein II or III apparatus with a Bio-Rad 1000/ 500 power supply were used to electrophorese the gels. Proteins were separated at 140-160 V for 1-1.5 h using SDS-PAGE running buffer (0.1 M Tris base, 0.1 M glycine, 0.5% (w/v) (SDS). Gels were stained overnight by shaking in Coomassie R Brilliant Blue. The gel background was destained in 10% (v/v) acetic acid. Prior to imaging, gels were replaced into a final destain solution which contained 4% (v/v) glycerol for 30 min to prevent cracking upon drying. Gels were imaged using a Canon 9950 f image scanner.

RESULTS

The effect of pH on the aggregation of beta-lactoglobulin: It has been reported previously that increasing pH increases the rate of aggregation and precipitation of heated beta-lactoglobulin[9,23]. Accordingly, the effect of pH on the rate of aggregation and precipitation of beta-lactoglobulin was studied by heating the sample at 70°C and at different pH values between 6.5 and 8.0. As pH increased, the rate of aggregation of beta-lactoglobulin also increased (Fig. 1, Table 1).

The chaperone action of alpha-crystallin in preventing aggregation of heated beta-lactoglobulin at different pH values.
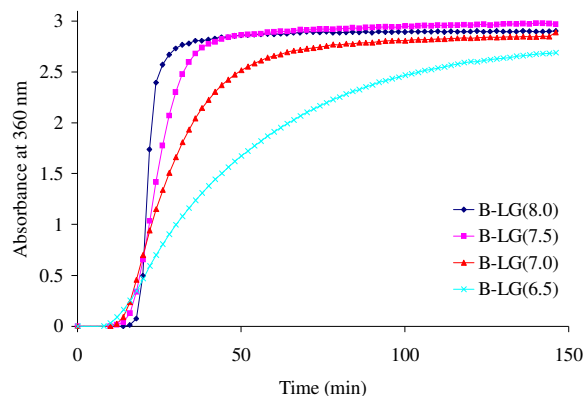

Fig. 1: Heat induced aggregation of beta-lactoglobulin at 5 mg mL^{-1} and different pH values. Protein was in total volume of 1 ml of 50 mM phosphate buffer, 0.05% NaN$_3$, 100 mM NaCl and 2.5 mM EDTA and the incubation temperature was 70°C

Table 1: Summary of rate constants for aggregation of heat-stressed beta-lactoglobulin at different pH values. The standard deviation obtained from Sigmaplot upon regeneration calculation

β-lactoglobulin	Rate constants×10^{-1} (min^{-1})
pH (8.0)	4.37±0.45
pH (7.5)	1.20±0.17
pH (7.0)	0.99±0.34
pH (6.5)	0.47±0.09

The chaperone-like action of alpha-crystallin in preventing the aggregation of beta-lactoglobulin was examined at different pH values. As shown in Fig. 2, alpha-crystallin is able to prevent the aggregation of beta-lactoglobulin at all pH values tested (i.e., 6.5, 7.0, 7.5 and 8.0). The efficiency of Alpha-crystallin as a molecular chaperone increased with increasing pH as demonstrated by its greater protection of beta-lactoglobulin against aggregation. At pH 8.0 and at the time point of 178 min, the aggregation of beta-lactoglobulin was suppressed ~92.2% in the presence of a 1:1 (w:w) ratio of alpha-crystallin:beta-lactoglobulin. At pH values of 7.5, 7.0 and 6.5 at the same time point, Alpha-crystallin prevented the aggregation of beta-lactoglobulin by ~76%, ~72% and ~43% respectively.

SDS-PAGE electrophoresis of heated beta-lactoglobulin in the presence and absence of alpha-crystallin at pH 7.0 and 8.0 values: Pellets and supernatants of the protein solutions (after heat induced aggregation experiments) at pH values of 8.0 and 7.0 were examined by SDS-PAGE for the distribution protein (Fig. 3A). Bands corresponding to ~18 kDa in
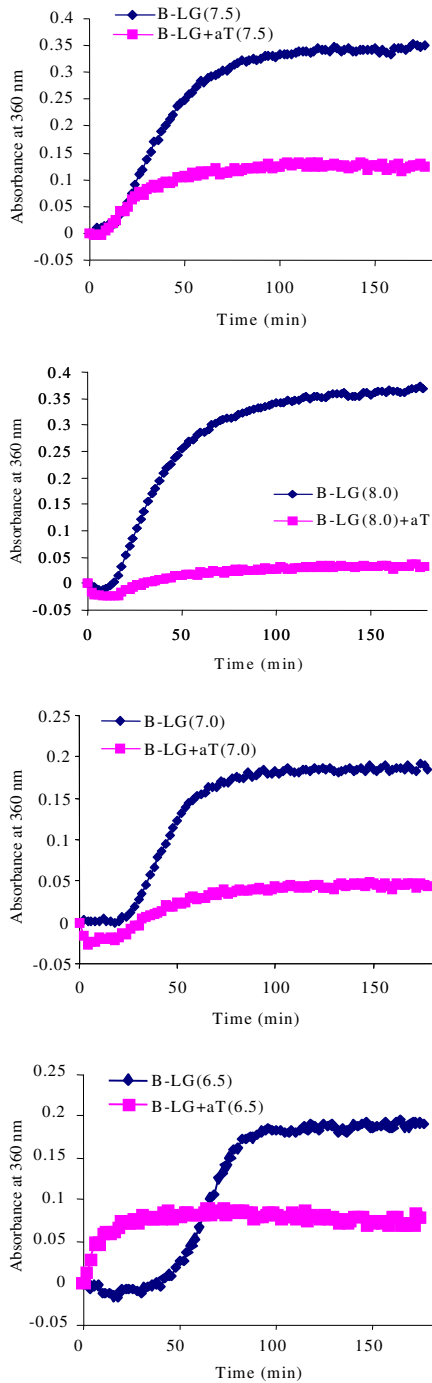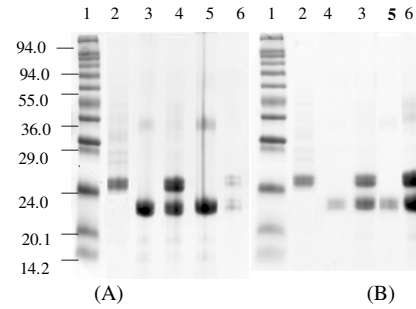
Fig. 3: SDS-PAGE gels showing the expression of (a) pellet and (b) supernatant of beta-lactoglobulin aggregation in the presence and absence of alpha-crystallin at pH 7.0 and 8.0 values. For both gels Lane 1: Molecular weight markers, Lane (2) alpha-crystallin, Lane (3): beta-lactoglobulin at pH 7.0, Lane (4): beta-lactoglobulin and alpha-crystallin at pH 7.0, Lane (5): beta-lactoglobulin at pH 8.0, Lane (6): beta-lactoglobulin and alpha-crystallin at pH 8.0

Fig. 2: Heat induced aggregation of beta-lactoglobulin at 5 mg mL^{-1} in the presence and absence of alpha-crystallin at different pH values. Protein was in total volume of 1 mL of 50 mM phosphate buffer, 0.05% NaN$_3$, 100 mM NaCl and 2.5 mM EDTA and the incubation temperature was 70°C

mass were observed at pH 7.0 and pH 8.0 both in the pellet and supernatant. Two bands corresponding to ~18 and 20 kDa in mass were also observed for beta-lactoglobulin and alpha-crystallin at pH 7.0 and pH 8.0 both in the pellet and supernatant. The strong bands shown in Lanes 3 and 5 in Fig. 3A show the precipitation of beta-lactoglobulin at pH 7.0 and 8.0 values. In the supernatant the bands are very weak (Fig. 3B) indicating that beta-lactoglobulin mostly precipitated under these conditions when heated. Lanes 4 and 6 show the bands of beta-lactoglobulin and alpha-crystallin at pH 7.0 and pH 8.0, respectively. At pH 7.0, the intensity of the bands are stronger in the pellet compared to the supernatant. This means that most of protein precipitated and alpha-crystallin was not effective in preventing the precipitation of beta-lactoglobulin. In fact, alpha-crystallin and beta-lactoglobulin co precipitated indicating that alpha-crystallin itself was destabilized under these conditions. At pH 8.0, very faint bands were present in the pellet suggesting little precipitation while very strong bands in the supernatant are consistent with both proteins being present. This means that at pH 8.0, alpha-crystallin was successful in forming a soluble complex with beta-lactoglobulin to prevent its aggregation and eventual precipitation. Thus, these data are consistent with alpha-crystallin being a more efficient chaperone at higher pH values as observed in the chaperone assay (Fig. 2).
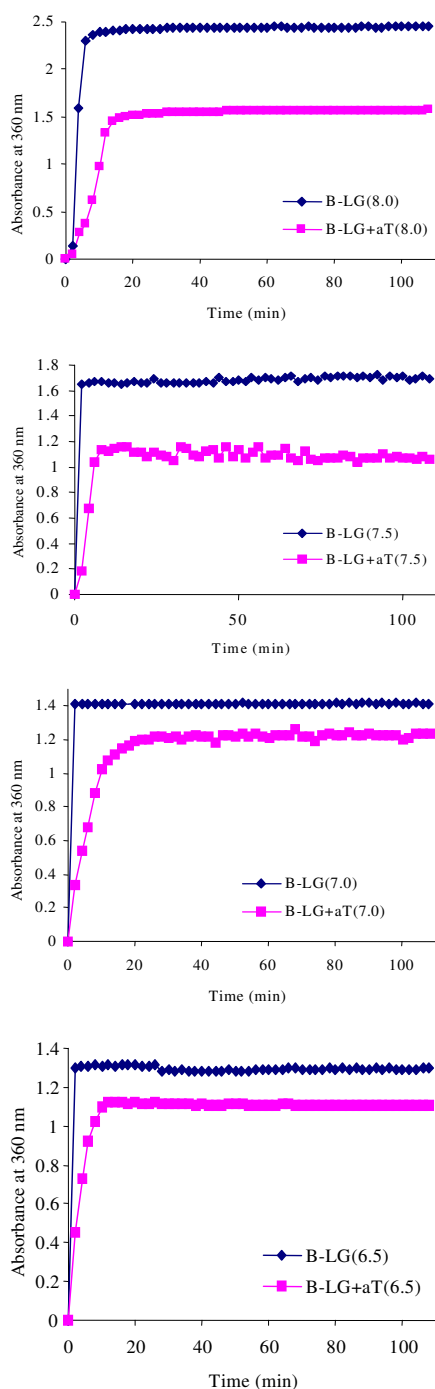
Fig. 4: Heat induced aggregation of reduced beta-lactoglobulin at 5 mg mL^{-1} in the presence and absence of alpha-crystallin and DTT at different pH values. Protein was in total volume of 1 mL of 50 mM phosphate buffer, 0.05% NaN$_3$, 100 mM NaCl, 2.5 mM EDTA and 20 mM DTT and the incubation temperature was 70°C

Table 2: Summary of rate constants for the aggregation of reduced and heated beta-lactoglobulin at different pH values. The standard deviation obtained from Sigmaplot upon regeneration calculation

β-lactoglobulin	Rate constants×10^{-1} (min^{-1})
pH (8.0)	5.71±0.30
pH (7.5)	1.50±1.5
pH (7.0)	1.01±0.54
pH (6.5)	1.47±1.1

Chaperone action of alpha-crystallin in preventing the aggregation of reduced and heated beta-lactoglobulin at different pH values: The chaperone-like action of alpha-crystallin in preventing aggregation of reduced and heated beta-lactoglobulin was examined at different pH values to compare the chaperone activity of alpha-crystallin with the non-reduced form of beta-lactoglobulin (Fig. 4). With increasing pH, the aggregation of reduced beta-lactoglobulin increased even more than in the absence of DTT (compare Table 2 with Table 1). The efficiency of alpha-crystallin as a molecular chaperone decreased in the presence of DTT as demonstrated by its poorer protection of beta-lactoglobulin against aggregation. At pH 8.0 and the time point of 100 min, the aggregation of beta-lactoglobulin was suppressed by approximately 36% with a 1:1 ratio (w:w) of alpha-crystallin to beta-lactoglobulin. At pH values of 7.5, 7.0 and 6.5 at the same time point, alpha-crystallin prevented the aggregation of beta-lactoglobulin by approximately 34, 15 and 14% respectively. These results show that DTT increased the rate of aggregation of heated beta-lactoglobulin and in this situation alpha-crystallin is a less efficient chaperone in preventing the aggregation of beta-lactoglobulin. Furthermore, the chaperone ability of alpha-crystallin increased with increasing pH. This is consistent with other data showing that the chaperone ability of alpha-crystallin is less efficient for rapidly aggregating target protein[24,25].

DISCUSSION

Beta-lactoglobulin aggregates via a combination of disulphide bond exchange (which leads to polymerization) and a nucleation-dependent mechanism due to hydrophobic association[26,27]. Mainly heat-induced aggregation of beta-lactoglobulin occurs through the formation of a dimer-monomer transition state of the protein. This transition state is characterized by a decrease in charge on the histidine residues of beta-lactoglobulin and an increased number of free thiol groups which in turn increases intermolecular thiol/disulfide linkage[23].

At pH values from 6.5-8.0, the aggregation and eventual precipitation of beta-lactoglobulin was investigated. The rate of aggregation of heat-stressed beta-lactoglobulin increased with increasing pH, which is consistent with the pH dependence of disulphide bond cleavage[23,26]. Law *et al*.[23] found that the rate of denaturation of beta-lactoglobulin was increased at higher pH values. A possible reason for this is that by increasing pH, the net negative charge on the protein also increases, which in turn encourages intramolecular electrostatic repulsion. In order to counteract this increased charge, dissociation from the dimer to the monomer form of beta-lactoglobulin is favoured[23,28]. This also increases the activity of thiol groups and hence their tendency to form covalent bonds, which eventually leads to unfolding and aggregation of, heated beta-lactoglobulin[23,28].

The effect of the molecular chaperone, alpha$_s$-casein, on preventing the aggregation of one of its natural targets, heat-stressed beta-lactoglobulin, has been previously demonstrated[29], whereby Alpha$_s$-casein effectively suppressed thermally induced aggregation of beta-lactoglobulin at 70°C and pH 7.0 and this chaperone ability of alpha$_s$-casein decreased with increasing pH which is the opposite in fact seen for alpha-crystallin. Devlin *et al*.[21] observed that alpha-crystallin was not able to prevent the aggregation of serpin (AT), which aggregates via a polymerization mechanism rather than a nucleation-dependent mechanism. However, alpha-crystallin inhibited the aggregation of a serpin (ACT), which aggregated via a nucleation-dependent mechanism. Thus, would alpha-crystallin inhibit beta-lactoglobulin aggregation and if so, would this decrease as the pH increased and via aggregation polymerization predominated? To answer these questions the chaperone action of alpha-crystallin was investigated in preventing the aggregation of heat stress beta-lactoglobulin at different pH values to determine its efficiency. It was found that at all pH values tested, alpha-crystallin prevented the aggregation of heat-stressed beta-lactoglobulin and this effect increased with increasing pH. Incomplete suppression of protein aggregation and precipitation by alpha-crystallin implies that the aggregation of beta-lactoglobulin occurs as a result of intermolecular disulphide bond polymerization. It has also been demonstrated that the chaperone action of Alpha-crystallin increased with increasing pH[30]. At lower pH values, the aggregation of beta-lactoglobulin occurs more by hydrophobic (nucleation-dependent) mechanisms since disulphide bond cleavage is highly pH dependent (i.e., it is reduced at lower pH values)[31,32]. Thus, it was proposed that even though

alpha-crystallin's chaperone action is reduced at lower pH values, it should be able to prevent beta-lactoglobulin aggregation better at lower rather than at higher pH values. In fact, in this study it was found that despite increasing hydrophobic aggregation of beta-lactoglobulin at lower pH, alpha-crystallin was a better chaperone at higher pH. In agreement with this, SDS-PAGE results confirmed the better chaperone action of alpha-crystallin at higher pH. These results imply that the increase in polymerization of beta-lactoglobulin at higher pH and the inability of alpha-crystallin to suppress this is offset by the enhanced chaperone ability of alpha-crystallin to suppress nucleation-dependent aggregation at higher pH.

Aggregation of beta-lactoglobulin occurs via both hydrophobic interactions and thiol/disulphide exchange reactions[26,27]. Increased aggregation of heated beta-lactoglobulin in the presence of DTT showed that aggregation of beta-lactoglobulin occurs both via intramolecular and intermolecular disulphide bond formation (hydrophobic interactions and a nucleation-dependent). Under these conditions beta-lactoglobulin aggregates much faster and alpha-crystallin is not a good chaperone[25] to prevent its aggregation.

At room temperature and physiological pH, beta-lactoglobulin exists as a dimer in which the monomers are noncovalently linked but dissociates into monomers upon heating[33]. beta-lactoglobulin undergoes heat-induced structural changes, which involve the exposure of buried hydrophobic groups and thiol groups[34].

By contrast[32] provided evidence that polymerization of beta-lactoglobulin into a gel network does not occur via disulphide linkages. They explain that disulphide linkages occur initially during heating and the association of the soluble aggregates into a network occurs by non-covalent bonding which is a hydrophobic and/or hydrogen bond interaction. The aggregation of beta-lactoglobulin can be described using a kinetic model[14]. In this kinetic model, the aggregation of beta-lactoglobulin is described by analogy with a radical-addition polymerization and consists of three steps. In the initiation step, the beta-lactoglobulin dimer splits into monomers, a process in which reversible aggregation is followed by an irreversible step, which is the real initiation reaction. This real initiation is a first-order reaction and the conformation of beta-lactoglobulin changes in such way that the free thiol group becomes reactive. This reactive thiol group reacts via thiol/disulphide exchange. The polymerization process stops when two reactive intermediates react with each other, forming a polymer without a reactive thiol group[14]. According to these data, at all pH values tested, aggregation of beta-

lactoglobulin occurs via both intermolecular exchange and hydrophobic interaction as showed in this study by the increasing aggregation of beta-lactoglobulin in the presence of DTT. It appears that increasing chaperone action of alpha-crystallin at higher pH rather than the hydrophobic aggregation by beta-lactoglobulin at lower pH is the predominant force for the chaperone action of alpha-crystallin. One explanation is that by increasing pH, the chaperone action of alpha-crystallin increases, which prevents both hydrophobic aggregation and non-covalent association of beta-lactoglobulin into a gel network. Another possibility could be that alpha-crystallin reverses the dimer-dissociation of heat-induced aggregation of beta-lactoglobulin, which is a necessary step in the aggregation process of beta-lactoglobulin[13].

CONCLUSION

This study reveals that upon heating, beta-lactoglobulin aggregation increased with increasing pH. As shown by light scattering profiles, the presence of DTT led to more rapid aggregation and precipitation of beta-lactoglobulin. Increasing aggregation of beta-lactoglobulin occurred in the presence of DTT implying that the aggregation of beta-lactoglobulin occurs via both intra and intermolecular disulphide bond formation. Alpha-crystallin prevented the aggregation of heat-stressed beta-lactoglobulin and was a more efficient chaperone at higher pH values. In the presence of DTT, however, alpha-crystallin was a less efficient chaperone due to faster aggregation of heated and reduced beta-lactoglobulin.

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