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The Effect of Polyethylene Glycol Induced Molecular Crowding on β-lactoglobulin Aggregation

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ABSTRACT

In vitro protein aggregation is affected by many different factors such as the presence of crowder agents. As we know the intracellular environment is highly crowded and it contains high concentrations of macromolecules. Molecular crowding decreases the effective volume available for the proteins and affects protein-protein interactions like protein aggregation. Aggregation of proteins may lead to conformational changes and consequent conformational diseases. It is possible to mimic crowding condition *in vitro* by adding inert molecule such as polyethylene glycol (PEG). In this study, the effect of different concentrations of PEG was evaluated on β-lactoglobulin (BLG) aggregation at different pHs. It was also aimed to see if the environmental factor like pH could affect the protein aggregation. BLG aggregation was detected by UV-Vis spectroscopy. The protein conformational changes were also examined by spectrofluorometer. SDS-PAGE method was applied to verify the disulfide bonds involvement in BLG aggregation. According to UV-Vis spectroscopy data, BLG absorbance increased at higher concentrations of PEG. PEG induced aggregation was also influenced by physical parameter pH. Based on fluorescence results, PEG affected BLG compactness. SDS-PAGE showed that increasing of protein concentration induces more chemical aggregation. As a conclusion, crowding agent, PEG, induces protein aggregation and pH affects this process. This causes protein conformational destruction and may alter BLG function.

Keywords: Macromolecular crowding, β-lactoglobulin, Aggregation, Polyethylene glycol

INTRODUCTION

Different physical and chemical factors including pH, salt and polymers influence protein folding and aggregation. Previous studies showed that the presence of polymers affect the behavior of the proteins upon interaction changes between protein and the polymer. This is also called molecular crowding [1]. As we know, cellular medium is crowded and contains high concentrations of macromolecules, such as proteins, nucleic acids, and carbohydrates which occupy up to 30% of the total cellular volume [2-4]. Presence of these macromolecules decreases the effective volume available for the other molecules in the cell, influences on biological phenomena by altering qualitative and quantitative properties of the molecules. It also affects macromolecules association, structure and stability of DNA, enzyme reactions, and cell volume regulation [5-7]. Molecular crowding has an important role in biological processes that occurs in the cell such as mechanism control. Therefore the investigation of this kind of biochemical processes *in vivo* differs from *in vitro* conditions. Since these conditions are a ubiquitous property of all cells, it is interesting to model crowding conditions in vitro and examine the effects [8-10].

It has been known that macromolecular crowding has a significant effect on protein-protein interactions such as protein aggregation. Protein aggregation is a usual biological process and occurs in proteins that partially unfolds or misfolds [11-13]. Protein misfolding and subsequent self-association (aggregation) results from conformational transition in protein structures. This may lead to conformational disease [14]. Thiol-disulphide bond interchange can cause protein association which is intermediated through hydrophobic interactions. Protein

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aggregates are usually toxic and associated with a variety of human diseases such as Alzheimer's, Parkinson's, Huntington's diseases and type II diabetes [15-17].

Molecular crowding tends to maintenance and stabilization of compact protein conformation. The protein conformation can be either on the pathway of folding or aggregation depending on the protein nature and the assay conditions [11,18]. Polyethylene glycol (PEG), dextran and ficoll (copolymer of sucrose and epichlorohydrin) are flexible hydrophilic polymers which are commonly used in aggregation studies induced by crowding agents [19]. Due to their compact and largely spherical shape, these polymers have relatively small surface to volume ratio. The property of relative hydrophilicity minimizes their specific interactions with proteins. Thus these polymers are believed to act primarily via excluded volume effect by decreasing the effective volume available for the proteins in the medium and increasing the effective protein concentration [20-23]. It is also mentioned that soft interactions including van der Waals, electrostatic and hydrophobic play an important role in crowding agent-protein behavior. The properties of the solvent are affected by the high concentration of crowding agent up to 40% *via* these interactions. This means that folding and aggregation of the protein is extremely sensitive to biopolymer existence in the medium [1].

It has been demonstrated that in the case of some proteins like lysozyme or superoxide dismutase with highly stable folded native states, addition of crowding agents causes the inhibition of the aggregation [24]. Some investigations have been shown that physical parameters such as protein structure and solvent pH can affect the aggregation of the protein in crowded condition. For example in the presence of crowding agent the aggregation of insulin in hexameric form (pH 7.5) was inhibited. It was increased in monomeric and dimeric form (pH 2.5). However, the aggregation of many proteins and peptides is accelerated in the presence of dextrans and other neutral flexible crowding agents [20]. Self-association of BLG depends on electrostatic interactions. This indicates the important role of ionic strength and solvent pH in protein self-assembly and consequent aggregation. Thus, the effect of crowding agents on protein aggregation mostly depends on the nature of the protein and also the environmental conditions [25].

In this research, we selected β-lactoglobulin (BLG) as target protein. BLG, the most abundant bovine whey protein, consists of 162 residues in monomeric form with a molecular weight of 18.3 kDa. The protein contains two disulfide bonds (cys160-cys66 and cys119-cys106) and one free cysteine residue (cys121). This free thiol group is localized in the center of BLG structure and takes part in the protein stability [26-29]. BLG exists in non-covalent dimer form at native conditions and it is currently proposed that there is a rapid equilibrium between native dimer and monomer form. The importance of BLG is due to its domination in overall aggregation of whey proteins. It is assumed that oligomerization of BLG may be affected by pH [30]. The conformation of BLG has been determined by X-ray crystallography and NMR spectroscopy. The secondary structure of BLG is composed of nine antiparallel β strands (A-I) and one major helix at the C-terminal end of the molecules [31-33]. BLG is determined to possess approximately 50% beta sheet, 15% alpha helix and 15-12% reverse turn structure [29].

BLG is a popular model protein and has received much attention in the matter of research. The aim of this study is to investigate the effects of PEG as a generally used macromolecular crowding agent on BLG conformation and aggregation. The crowded condition was examined at different pH conditions of the solvent. This was to find out BLG aggregation manner induced by physical parameters such as crowding agent concentration and solvent pH.

MATERIAL AND METHODS

Materials

PEG 8000 Da, Bovine β-lactoglobulin (L3908) and all other chemicals were from Sigma. Acrylamide and bisacrylamide were purchased from Titran Company. Distilled water was used to prepare experiment solutions.

Methods

Sample preparation. A) Sample preparation for concentration related experiment. BLG was dissolved in Tris buffer 20 mM containing 60 mM NaCl, pH 7 [34] in the presence of different PEG concentrations (0, 5, 10, 15 and 20%). Samples were incubated at 37 °C for 1 h. Then

cooled at room temperature and used for detection experiments.

B) Sample preparation for pH related experiment. 1 mg ml^{-1} BLG was dissolved in Tris buffer 20 mM containing 60 mM NaCl and incubated at 37 °C [33] for 1 h at different pH 2, 5, 7 and 8. Measurements were done at 25 °C.

Turbidity measurement. In order to compare the solubility of protein samples in the presence and absence of additives, turbidity measurements were done at 600 nm [35].

The degree of BLG aggregation was quantitatively evaluated by measuring sample turbidity using UV-Vis spectrophotometer (CamSpec M510). To do this, 1 mg ml^{-1} BLG was dissolved in Tris buffer and incubated at physiological condition (pH 7, 37 °C) for 1 h in the presence and absence of different concentrations of PEG. Protein/polymer mole ratio is approximately (9:1), (4:1), (3:1) and (2:1) respectively for 5 to 20% PEG. The optical density of protein samples was obtained at 600 nm. The data gathered after 3 times repetition.

Light scattering measurement. Another method to evaluate the occurrence of aggregates is to measure optical density at 340-360 nm [36]. In this study, 0.05 mg ml^{-1} protein containing samples were prepared in the presence of different PEG concentrations (0, 5, 10, 15, and 20%) and at different pHs (2, 5, 7, and 8). Polymer/protein mole ratio is approximately (2:1), (5:1), (7:1) and (9:1), respectively for 5 to 20% PEG. After 1 h incubation at 37 °C, they cooled to room temperature. The excitation and emission wavelengths were set to 350 nm. Triplicate measurements were done for each data.

Fluorescence spectroscopy. Examination of tryptophan intrinsic fluorescence helps us understand the conformation and association of BLG. Aromatic side chains of amino acids such as tryptophan and tyrosine are the intrinsic fluorophores in proteins. In the case of BLG fluorescence intensity is dominated by tryptophan. Emission wavelength could be affected by change of protein conformation. Alteration of tryptophan residues environment may cause the conformational change of the protein. Polarity of the surrounding environment induces these alterations. In order to measure intrinsic intensity of BLG fluorescence, the samples were excited around 280 nm and the emission was gathered between 300 to 500 nm [28]. Based on previous studies, we examined the fluorescence intensity of samples in the way as follows.

Intrinsic fluorescence of the protein samples was measured at room temperature using Varian Cary Eclipse fluorescence spectrophotometer in a quartz cell of 1 cm path length in the desired buffer. Protein concentration was 0.05 mg ml^{-1} concentration. Polymer/protein mole ratio is approximately (2:1), (5:1), (7:1) and (9:1), respectively for 5 to 20% PEG. The excitation wavelengths were 280 and 295 nm and emission was recorded between 300-450 nm. Bandwidths were set at 5 for both excitation and emission.

SDS-PAGE. The samples were analyzed by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE). SDS-PAGE of the native and heated BLG was performed using running gel of 15%. Samples were prepared at pH 7, incubated at 37 °C for 1 h and cooled to room temperature. The samples were diluted with SDS-PAGE sample buffer. After 5 min boiling, 50 µl of each sample was loaded into the sample slots to precisely compare intensity of the protein bands. At last the samples were stained [32,34,37].

RESULTS AND DISCUSSION

Measurement of Turbidity

The result of protein turbidity is shown in Fig. 1. As shown, no considerable aggregation detected at 10% PEG and the concentrations below that, but it was increased at higher concentrations and highest concentration of PEG induced more aggregation. In addition, the results imply that at pH 2, the turbidity of the samples is substantially less than the other pH values indicating aggregation of BLG is weak on this condition and PEG doesn't strengthen it. In contrast, vast majority of aggregated protein was detected at pH 5. This may perhaps be related to the isoelectric point (pI) of BLG. It has been investigated that as a generic ability, spherical aggregates of proteins are formed at or near the pI [38- 40]. The pI value of the proteins varies related to the protein source and the state. pI value of BLG is mostly considered as around pH 5.1 [38,40,41]. Consistent with this criterion, high amount of aggregation was revealed when BLG was incubated at pH 5.

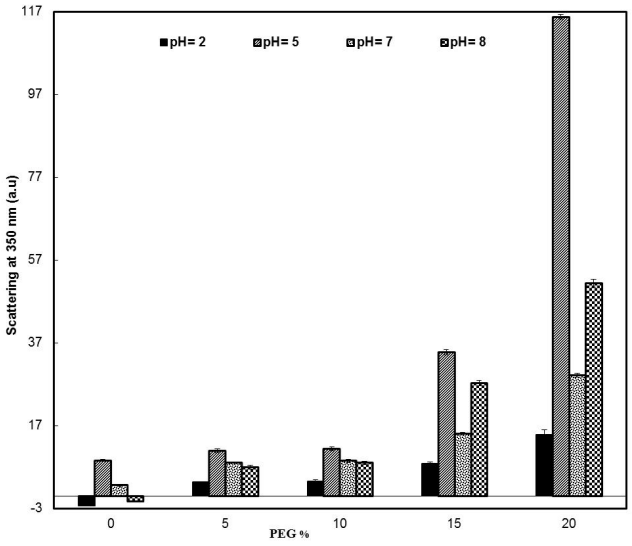

Fig. 1. Turbidity (absorbance at 600 nm) of BLG solutions at pHs 2, 5, 7 and 8 in the presence of different PEG concentrations (0, 5, 10, 15 and 20%). All samples were heated at 37 °C for 1 h and the absorbance was measured after the samples were cooled.(——) pH 2; (….) pH 5; (---) pH 7; (— —) pH 8.

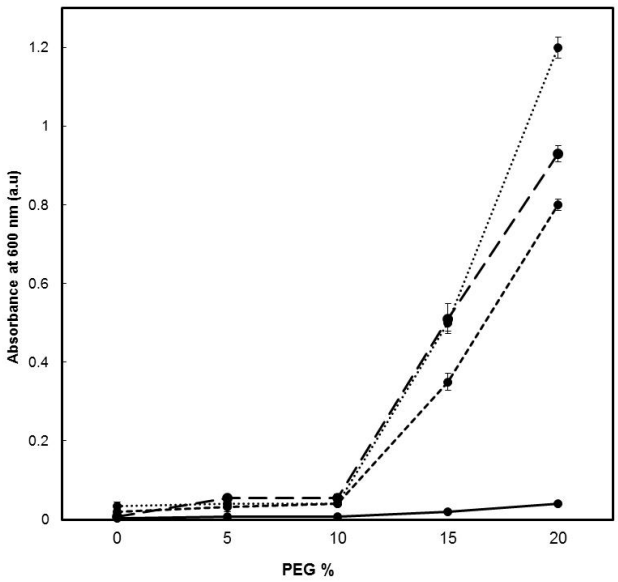

Fig. 2. Light scattering measured at 350 nm for samples with a protein concentration of 0.05 mg ml^{-1}. BLG solutions at pHs 2, 5, 7 and 8 in the presence of different PEG concentrations (0, 5, 10, 15 and 20%).

Measurement of Light Scattering

Figure 2 shows the amount of scattered light at 350 nm. These data are consistent with turbidity results which show negligible aggregation at low concentrations of PEG (5 and 10%). BLG aggregation was slightly increased by PEG addition (0-20%). However the vast amount of aggregated species was detected at high PEG concentration. The differences among the samples are more obvious in fluorescence spectroscopy in comparison with absorption spectroscopy mentioned as follows. In addition, their difference becomes clearer at 15% and 20% PEG. The aggregation of BLG at various pH values is as follow: pH 5 > pH 8 > pH 7 > pH 2.

Fluorescence Spectroscopy

Fluorescence spectroscopy can provide valuable information for local environments of aromatic amino acid residues and it is sensitive to conformational changes of proteins [42,43]. BLG contains two tryptophan residues, Trp 19 and Trp 61. Trp 19 is buried inside the protein, but Trp 61 is solvent exposed. In dimeric form, two Trp 61 residues come near each other and fluorescence quenching occurs. Furthermore there is a disulphide bond (Cys 66 and Cys 160) in BLG structure which acts as a quencher for Trp 61 in monomeric form. So the fluorescence intensity of BLG mainly refers to Trp 19.

Intrinsic fluorescence spectra of BLG excited at 280 nm and 295 nm are respectively shown in Figs. 2 and 3. The spectra are recorded at pH 2.0, 4.7, 7.0 and 8.0.Trp, Tyr and Phe excited at 280 nm so the emission spectra are due to all of them, while Trp is the only residue excited at 295 nm. As shown, intensity of the emission spectra increases after addition of PEG and there is almost a direct relation between intensity and concentration of PEG at both exciting wavelengths. Our pervious investigation [44] also indicated that aggregation of BLG due to addition of trifluoroethanol (TFE) results in the similar change in the intensity of fluorescence spectra. This change is probably due to increasing the distance of Trp 19 and Cys66–Cys160 bond. As shown in Figs. 3 and 4 no meaningful shift is observed in the spectra upon addition of PEG, however TFE causes the spectra shift to the longer wavelength. We guess that the structural transition from β-sheet to α-helix results in the

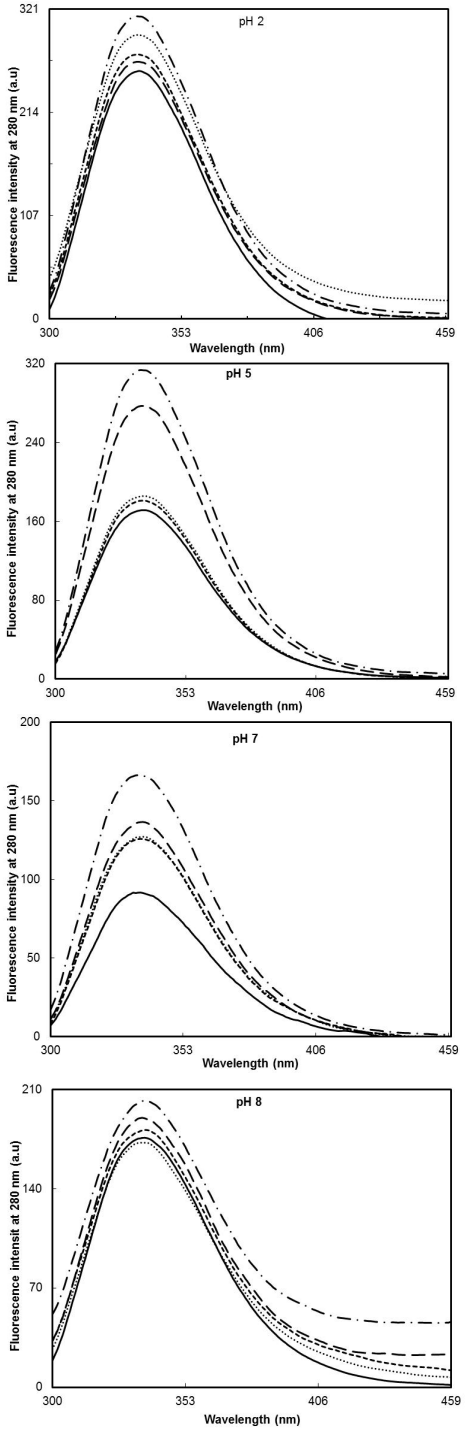

Fig. 3. Emission spectra of 0.05 mg ml^{-1} native BLG, solutions were prepared at pHs 2, 5, 7 and 8, in the presence of different concentrations of PEG (0, 5, 10, 15 and 20 %). All samples were excited at 280 nm. (—) 0% PEG; (…) 5% PEG; (---) 10% PEG; (— —) 15% PEG; (— • —) 20% PEG.

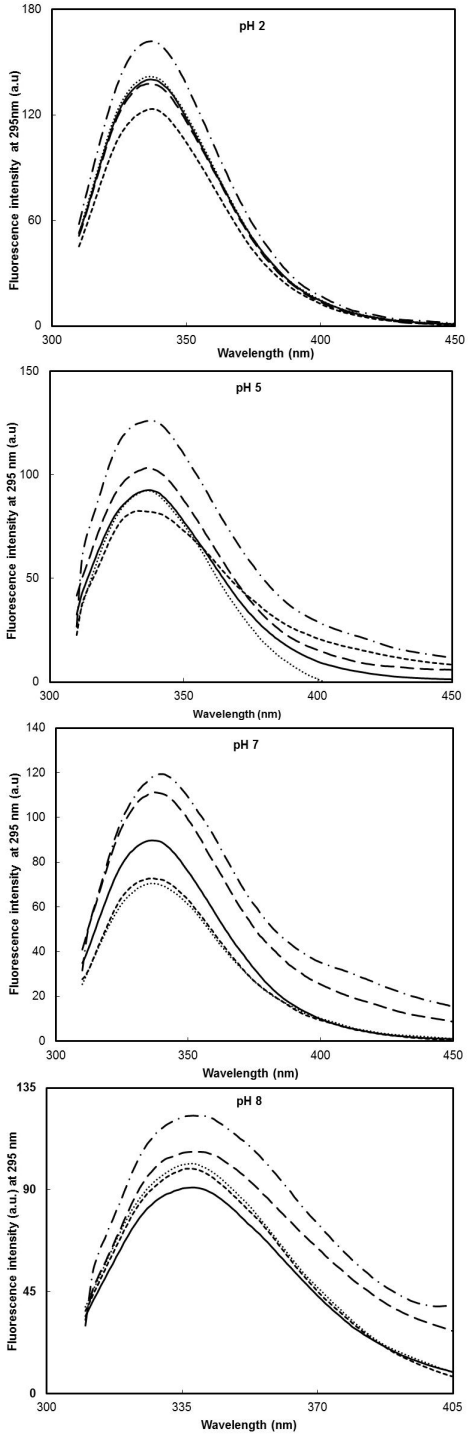

Fig. 4. Emission spectra of 0.05 mg ml^{-1} native BLG. Solutions were prepared at pHs 2, 5, 7 and 8,in the presence of differentconcentrations of PEG (0, 5, 10, 15 and 20 %). All samples were excited at 295 nm. (—) 0% PEG; (…) 5% PEG; (---) 10% PEG; (— —) 15% PEG; (— • —) 20% PEG.

observed red-shift. The pervious result implied that the transition in the secondary structure of BLG occurs in presence of TFE, but the result of circular dichroism indicated that PEG doesn't change the type of the secondary structure (supplementary data, Fig. 1S). In the presence of 15% PEG, at pH 7, intensity of circular dichroism spectra decreases slightly indicating a little change in the compactness of the secondary structure. According to the spectroscopic results, we can guess that PEG influences on the compactness of the protein structure, but doesn't disrupt it.

In accordance with the data from absorbance experiments (Figs. 1 and 2) it is clear that the aggregation of BLG is promoted in the presence of PEG. Furthermore PEG causes BLG conformational changes as confirmed by fluorescence experiments (Figs. 3 and 4). So the hydrophobic patches of BLG are exposed to the solvent and aggregation happens. Different physical parameters such as protein concentration and pH affect this aggregation as mentioned in results. These factors may change the net charge of BLG and reduction in net charge mostly results in amorphous aggregates [38].

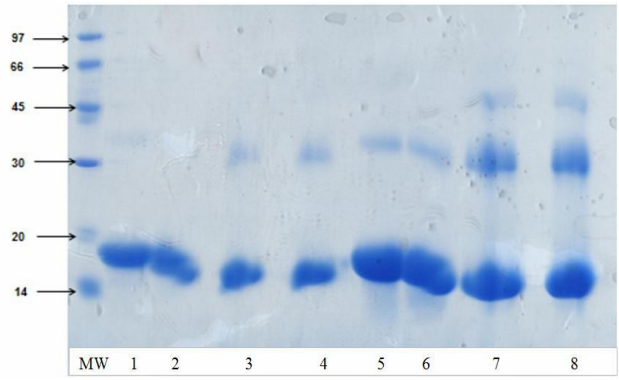

Fig. 5. SDS-PAGE of BLG in presence 15% PEG, pH 7: 1 and 2: BLG 0.5 mg ml^{-1} in the absence of 15% PEG (reducing, and non-reducing conditions, respectively). 3 and 4: BLG 0.5 mg ml^{-1} in the presence of 15% PEG (non-reducing conditions before and after heating respectively). 5 and 6: BLG 2 mg ml^{-1} in the absence of 15% PEG (reducing, and non-reducing conditions respectively). 7 and 8: BLG 2 mg ml^{-1} in the presence of 15% PEG (non-reducing conditions before and after heating respectively).

SDS-PAGE

SDS-PAGE is an analytical tool with the ability to detect the covalent nature of aggregates. For noncovalent association of the protein species, separated polypeptide chains will be assessed [36]. SDS-PAGE result of BLG is shown in Fig. 5. The experiments were carried out in both reducing and non-reducing conditions to verify that disulfide bond formation is involved in the aggregation of BLG. Aggregated BLG linked through disulphide bonds remains intact under non-reducing conditions and oligomer bands are appeared. Non-aggregated and non-covalently aggregated forms of BLG are observed in monomer band. Furthermore, in order to reach a conclusion SDS-PAGE of BLG was examined at two different BLG concentrations (0.5 and 2 mg ml^{-1}) and 15% PEG as an optimized concentration which resembles crowded condition.

Native BLG (0.5 mg ml^{-1}) in reducing and non-reducing conditions is loaded in wells 1 and 2, respectively and show the monomeric state of the protein. Wells 3 and 4 contain 0.5 mg ml^{-1} BLG in the presence of 15% PEG at non-reducing conditions before and after heating, respectively. The image shows sharp monomer band. Furthermore slight dimer band is seen. It means that PEG is able to induce monomer to dimer conversion. But dimer band is not so strong because of low concentration of BLG. Wells 5 and 6 shows the result for 2 mg ml^{-1} BLG in the absence of PEG in reducing and non-reducing conditions. The strong monomer and week dimer bands are visible. It is clear that the increase in BLG concentration promotes dimer formation as compared to 0.5 mg ml^{-1} BLG by similar treatment condition (wells 1 and 2). Wells 7 and 8 contain 2 mg ml^{-1} BLG in the presence of PEG at non-reducing conditions before and after heating, respectively. Three bands are apparent in the image which stands for monomer, dimer and trimer states of the protein. So BLG is able to form dimer and also trimer in the presence of PEG at higher concentration. On the whole, the results show that chemical aggregation of BLG is in close relationship with protein concentration; increasing the protein concentration induces more chemical aggregation (monomer to dimer transition). It is also clear that molecular crowding can promote the

aggregation process. It may be concluded that BLG aggregates are covalently bond and resistant to reducing agents. Because we see dimer forms of BLG at both reducing and non-reducing conditions (wells 5 and 6). So the only strategy to reduce BLG concentration is lowering protein concentration and reductants have no effect on the aggregation.

CONCLUSIONS

It is known that only correctly folded state of a protein is able to do its functions properly. However there are some factors that affect the proteins folding and most of the time trigger the aggregation. One of these conditions is cellular crowding. This study makes use of PEG which resembles the crowded condition of the cells. The results indicated that molecular crowding induced by PEG promotes aggregation of BLG at various pHs. The intensity of aggregation of BLG depends on pH and the most intensive aggregation is shown at pH 5. In addition, aggregation is accompanied with local changing of the protein structure. It is noteworthy to consider the importance of BLG aggregation in biochemical and biotechnological applications. BLG is a suitable carrier for nutrients and also good candidate for encapsulation of drugs since it is resistant to pepsin and secondly digested slowly by trypsin. Based on data taken here, it is suggested to give attention to physicochemical parameters affecting the efficiency of the protein. We know that pH and also BLG concentration induce the aggregation. Considering neutral pH, high amount of BLG will terminate in aggregation and disturb the conformation and may influence on delivery function. So finding out the optimum concentration will be of interest to reduce the side effects. However crowding the medium is noticeable. Although it is hard to preciously examine the crowding condition behavior, but data shows the increment of BLG aggregation in the presence of the crowder agent. Therefore a low concentration of BLG is more suitable for its delivery in crowded cell medium at neutral pH.

DECLARATIONS

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