Results & Discussion-I

3.1 Purification and characterization of bovine BLG

Beta-Lactoglobulin (BLG), a member of the lipocalin super family, is the dominant whey protein (Madureira et al., 2007) first characterized in 1934 by Palmer (1934). In spite of lack of assignment of definite biological function, the protein has been subjected to extensive studies for a variety of reasons. These include its potential role of BLG as a carrier of retinol and fatty acids (Liang et al., 2008), as a causative protein of cow’s milk allergy (Crittenden et al., 2005), as an excellent source of branched chain and other essential amino acids (Madureira et al., 2007) and a model protein with thoroughly studied three dimensional structure (Sawyer and Kontopidis, 2000). Whey has been widely used as a source of BLG because of its ready availability in bulk quantities as a by-product of curd and cheese manufacture (Smithers et al., 2008) and a number of procedures have been published on the BLG purification (Kinekawa and Kitabatake, 1996; Neyestani et al., 2003; Lozano et al., 2008).

3.1.1 Purification of BLG

We worked out a simple one step procedure for the purification BLG from bovine milk taking into consideration the ability of BLG to exist as a 18 kDa monomer at acid pH and the fractionation range of Bio-Gel P10 that could separate both large molecular weight proteins including casein and the α-LA with lower molecular weight. The schematic representation of the purification is shown in figure 3.1.1. Whey prepared as described in methods was loaded on a small Bio-Gel P10 column (2.3 cm x 83 cm) equilibrated with 20 mM phosphate buffer pH 3.0. The column was also eluted with the same buffer. A single run on the column yielded over 10 mg of BLG in about 4.0 hours. This is significant since Bio-Gel P10 offers considerable scope for scale up. As shown in figure 3.1.2, the elution profile revealed, besides the small peak (peak 1) in the void volume, two fully resolved and distinct peaks (peaks 2 and 3) of comparable peak heights. On SDS-PAGE, the eluant in the first peak migrated as a heterogeneous mixture of several high and medium molecular weight polypeptides, presumably corresponding to caseins and other proteins known to occur in whey (Madureira et al., 2007). The second and third peaks migrated as compact single bands (Fig. 3.1.2. inset).
Fig. 3.1.1: A schematic representation of the steps involved in the purification of BLG
Raw Milk
- pH 4.6 by 10% acetic acid
- Centrifuge at 10,000 rpm, 10 min

Whey (supernatant)
- pH 3.0 by 1M HCl
- Centrifuge at 10,000 rpm, 10 min

Whey at pH 3.0
- Dialysis against 20 mM phosphate buffer, pH 3.0 containing 18 mM NaCl

Gel filtration chromatography on Bio-Gel P10
- BLG
- α-LA

SDS & Native PAGE, Absorption spectroscopy, Fluorescence spectroscopy, Circular dichroism spectroscopy & western blotting
Fig. 3.1.2: Purification of BLG from bovine whey

Bovine milk whey was adjusted to pH 3.0 and loaded onto a Bio-Gel P10 column (2.3 cm x 83 cm) equilibrated with 20 mM phosphate buffer, pH 3.0 containing 18 mM NaCl. Elution of the column was performed with the same buffer and the fractions monitored at 280 nm.

Inset: SDS-PAGE of eluants from the gel filtration column. Electrophoresis was performed using 15 % (w/v) acrylamide gel. Lane 1, 2, 3 & 4 contain cow’s milk whey (30 μg), pooled void peak (peak 1) (20 μg), pooled peak 2 (20 μg) and pooled peak 3 (10 μg) samples, respectively. The gel was run according to the procedure of Laemmli (1970) and stained with CBB R-250 as described in the methods.
The Bio-Gel P10 column was calibrated using molecular weight marker proteins and the molecular weights of proteins that emerged in the peaks 2 and peak 3 were calculated to be \( \sim 18 \) kDa and \( \sim 14 \) kDa respectively (Fig 3.1.3 A, B). These values correspond to the molecular weights of bovine BLG and \( \alpha \)-LA respectively. Figure 3.1.4 (inset) shows SDS-PAGE of pooled peak 2 along with molecular weight markers and commercial BLG variants A and B. Similar to the commercial proteins, the molecular weight of the protein in peak 2 (Weber and Osborn, 1969) was 18.1 kDa (Fig 3.1.4) substantiating the molecular weight values obtained from gel filtration data. The commercial BLG variants A and B migrated with mobilities that were indistinguishable from that of the purified BLG (Fig. 3.1.4, inset).

Absence of additional bands in the gels that were loaded with 20 \( \mu \)g purified BLG suggests homogeneity of the protein. The protein in the third peak also migrated as a sharp band (Fig. 3.1.2, inset lane 4) exactly corresponding in molecular weight to \( \alpha \)-LA (Fig. 3.1.3) indicating that \( \alpha \)-LA is also purified simultaneously to homogeneity. As shown in the figure 3.1.5, the limit of detection of the Coomassie Blue staining lies, under the conditions used, between 0.1 and 1.0 \( \mu \)g of BLG. Since 20 \( \mu \)g protein was applied in the lane 3, we expect the purified BLG to be at least 95 percent pure. The faint slow migrating band visible in lanes loaded with >10 \( \mu \)g protein is apparently an aggregation product of BLG. It is well known that around neutral pH BLG aggregates in to dimers (Sawyer and Kontopidis, 2000).

The slight asymmetry of the peak 2 (Fig. 3.1.2), in spite of the apparent homogeneity evident in the SDS gels is difficult to explain. One possibility is that a small population of BLG molecules in whey may be modified with sugars contributing small differences in molecular dimensions and behavior during gel filtration. The observation of Chevalier et al. (2001) that about 20 percent of free amino groups of BLG are unavailable for reaction with orthophthalaldehyde, also suggests possibility of such modification.

Gel filtration was performed using phosphate buffer, pH 3.0, since BLG is known to occur predominantly in monomeric form at this pH (Morgan et al., 1997). The exclusion limit of Bio-Gel P10 is 20 kDa. Hence, traces of caseins remaining in the whey and other higher molecular weight whey proteins like BSA (68 kDa), immunoglobulins, lactoferrin (77 kDa) and lactoperoxidase (80 kDa) were excluded from the gel column and emerged from the column in the void volume (Fig. 3.1.2, peak
Fig. 3.1.3: Molecular weight determination of gel filtration peaks 2 and 3.

(A) Elution profiles of molecular weight standards and whey proteins emerging as peak 2 and 3 from a Bio-Gel P10 column (2.3 cm x 83 cm). Elution positions of molecular weight markers and peak 2 and 3 proteins have been shown.

- Bovine beta-lactoglobulin,
- Lysozyme,
- Cytochrome c,
- Insulin,
- Whey proteins BLG and α-LA

(B) Determination of molecular weight of peak 2 and 3 using the theoretical treatment of Andrews (1964) as detailed in method section. Molecular weight marker contained Insulin (IN), 5 kDa; Cytochrome c (CC), 12 kDa; lysozyme (LY), 14.3 kDa and bovine beta-lactoglobulin (BG), 18 kDa.
Void Volume: 90 ml
Bed Volume: 300 ml

Absorbance at 280 nm
Absorbance at 562 nm

Elution volume (ml)

Log M

Elution Volume/Void volume Ve/Vo

IN
CC
purified α-LA → LZ
purified BLG → BG
Fig. 3.1.4: **SDS-PAGE of purified BLG and determination of molecular weight**

The procedure of Weber & Osborn (1969) was used for the calculation of molecular weight from the mobility of the pooled peak 2 fraction eluted from the Bio-Gel column in SDS gels run in presence of 5% β-mercaptoethanol. Molecular weight markers used were phosphorylase b (PB), 97.4 kDa; bovine serum albumin (BSA), 66 kDa; ovalbumin (OA), 43 kDa; carbonic anhydrase (CA), 29 kDa, soyabean trypsin inhibitor (STI), 20.1 kDa and lysozyme (LY), 14.3 kDa.

**Inset-** SDS PAGE of BLG was performed using 12% (w/v) acrylamide gel according to the procedure of Laemmli (1970). Lanes 1, 2 and 3 contain 20 μg each of commercial BLG A, BLG B and peak 2 (BLG purified in this study) sample, respectively while lane M contains the molecular weight marker (Genei, medium range). The staining was performed with CBB R-250 as described in the methods.
Fig. 3.1.5: Sensitivity of CBB R-250 staining of BLG in reducing SDS-polyacrylamide gels.

(A) Purified BLG (0.1, 1.0, 3.0, 5.0, 10.0 and 20.0 µg) was loaded on the gel in the lanes 1-6, respectively and subjected to electrophoresis. Staining was performed with CBB R-250 as described in the methods.

Fig. 3.1.6: A comparison of the migration behavior of purified BLG with commercial bovine BLG variants A and B on non-denaturing PAGE.

Non-denaturing PAGE (15% w/v) and staining with CBB R-250 was performed as described in the methods. Lanes 1, 2 & 3 contain 20 µg each of commercial BLG variants A, BLG variant B and purified BLG, respectively. Lane 4 contains 20 µg of pooled peak 2 fractions eluted from the Bio-Gel column loaded with bovine milk whey mixed with commercial BLG A.
1), while the monomeric BLG and α-LA were resolved and emerged as separate peaks. While several variants of BLG from various mammals have been isolated, cow’s milk contains predominantly the variants A or B. The BLG isoforms A and B differ only in two amino acid residues. BLG A has Asp at position 64, while in variant B it is substituted with Gly. In addition, Val 118 of variant A is replaced with Ala in the isoform B (Sawyer and Kontopidis, 2000). The difference in the molecular weights of the two variants is too small for resolution on SDS-PAGE, but the two differ slightly in electrophoretic mobility in the native gels. The Native PAGE profile of BLG purified by the procedure described above revealed a band migrating as the variant B (Fig. 3.1.6). As discussed earlier aggregation of BLG preparation was evident in the native (non-SDS) gels. Interestingly predominance of B allele in the bovine milk in this part of the world is well known (Patel et al., 2007; Rachagani et al., 2006). In order to ascertain if the procedure described is also applicable in the purification of the variant A, when present, commercial bovine BLG A was added to the whey prior to gel filtration through the Bio-Gel column. When pooled peak 2 eluted from the column was subjected to native PAGE, band corresponding to both variant A and B was detectable in the gel (Fig 3.1.6, lane 4). This suggests that using the Bio-Gel P10 gel filtration procedure, both the major variants can be purified from other whey proteins. Additional procedures will however be required to resolve the variant mixture if desired.

The procedure of BLG purification described was also useful in the purification of BLG from buffalo whey. Bio-Gel P10 chromatography of the buffalo whey also yielded well resolved two peaks, in addition to that in the void volume, which on SDS-PAGE exhibited migration behavior comparable to BLG and α-LA (Fig 3.1.7).

### 3.1.2 Characterization of the purified BLG

In order to establish if the purified BLG B is indeed in native form, a comparison of its spectral properties with the commercial bovine BLG variant B was made. Almost identical absorption spectra (Fig. 3.1.8 A) were obtained with the purified and commercial BLG preparations showing an absorption maximum of ~278nm. Intrinsic fluorescence study also showed maximum emission intensity at ~331 nm after excitation at 280 nm, both for commercial BLG B and the purified protein (Fig. 3.1.8 B). These observations suggest that the native conformation of BLG is
Fig. 3.1.7: SDS-PAGE of pooled peaks 1, 2 and 3 emerging from the Bio-Gel P10 column loaded with buffalo milk whey.

Electrophoresis was performed using 15 % (w/v) acrylamide gel. Lane 1, 2, 3 & 4 contain buffalo whey pooled peaks 1, 2 (20 µg), and 3 (10 µg) respectively.
Fig. 3.1.8: A comparison of absorption (A), fluorescence (B) and far UV-CD spectra of purified and commercial BLG.

Absorption (A), fluorescence (B) and UV-CD (C) spectral properties of BLG purified using the procedure described and commercial protein. The proteins were dissolved in 10 mM Tris HCl, pH 7.0. Absorption measurements were taken between 230-310 nm (protein concentration; 0.35 mg mL\(^{-1}\)). For fluorescence measurements, the samples (0.1 mg mL\(^{-1}\)) were excited at 280 nm and emission measured between 300-450 nm. Samples (0.2 mg mL\(^{-1}\)) were scanned between 190-250 nm for far the UV-CD spectra.

( ) commercial BLG B

(---•) purified BLG
RESULTS & DISCUSSION

Retained during the purification. Far-UV CD spectrum (Fig. 3.1.8 C) of the purified protein with a maximum near 216 nm, similar to the commercial BLG B, indicates that the former contains predominantly native like β-sheet structure (Papiz et al., 1986).

Retention of the native conformation by the BLG during gel filtration at pH 3.0 was anticipated in view of the well known stability of protein at acid pH (Kella and Kinsella, 1988). It is because of its stability in acid pH that BLG is considered a potential carrier of fat soluble vitamins that bind to the native protein (Ragona et al., 2002). Strategies that minimally affect the native structure of BLG during purification have also received attention in the past (Ye et al., 2000; Neyestani et al., 2003). The two step procedure of BLG purification described more recently by Lozano et al. (2008) also yields the homogenous protein in native form, but compared to the present procedure it is more time consuming.

Antigenicity of the purified protein was also examined by Western blot analysis using the anti-BLG antibodies raised in rabbits against the purified protein (Fig. 3.1.9). The observed cross reactivity of the antibodies, also with commercial BLG A and B variants suggested the preservation of the antigenicity of the purified BLG presumably due to retention of native three dimensional structure. It also supports the earlier observations that BLG variants A and B share common epitopes and remarkable similarities in the conformation and 3-D structure as observed by several investigators (Monaco et al., 1987; Dong et al., 1996). Absence of immuno-staining at positions other than that of BLG in the lane containing the purified protein also supports the homogeneity of the BLG purified from cow's milk in this study.

As mentioned already, the marked differences between the molecular dimensions of BLG existing as monomer at acid pH and other whey proteins were exploited for the purification of the former in homogenous form by gel filtration through a Bio-Gel P10 column. Spectral and immunological studies suggest that the purified protein is almost indistinguishable from the Sigma bovine BLG variant B. The purification procedure also offers the attractive possibility of simultaneously purifying the second major whey protein α-LA and could be readily adapted for the purification of buffalo whey BLG. We have not however characterized the purified α-LA and further studies are needed to establish if the purified α-LA is in indeed native state especially considering the low pH conditions used in the experiment (Bramaud et al., 1995).
Fig. 3.1.9: Western blot analysis of the purified BLG

Rabbits were immunized with purified BLG and the IgG purified as described in the text. Details of SDS-PAGE, blotting on nitrocellulose membrane, and staining with HRP-conjugated secondary antibody were performed as described in Methods. Lane 1 contains the purified BLG, lane 2 commercial BLG A and lane 3 commercial BLG B.
Bio-Gel P10 beads are rigid and less compressible due to high level of cross-linking of the polyacrylamide gel. This together with their remarkable resistance to acid (Bio-Gel P, Polyacrylamide gel, Instruction Manual, Bio-Rad, CA), suggests the possibility of scaling up of the purification procedure. Moreover, Bio-Gel P gel is autoclavable and packed columns of Bio-Gel P gel can be stored indefinitely if maintained at neutral pH in the presence of a bacteriostat such as 0.02% sodium azide at 4 °C.

A simple strategy that yields good amount of purified BLG conveniently may contribute significantly in the study of the protein which, in spite of lack of an established physiological role, has been ascribed several interesting functions including those in the development of passive immunity (Sutton and Alston-Mills, 2006) and even protection against cancer (Mcintosh et al., 1995). In addition several bioactivities have been linked with the peptides derived from BLG. These include angiotensin-I-converting enzyme inhibition as well as antihypertensive, antioxidant, antimicrobial, opioid and hypocholesterolemic activities (Hernander-Ledesma et al., 2008). As has already been pointed out, nutritional value of BLG is well recognized and the protein has several actual and potential uses in modern foods and beverages dietary supplements, functional food and pharmaceutical preparations. To conclude, ready availability of adequate quantities of pure BLG may further the investigations on bioactivities of the component peptides of the protein.
Effect of heating with sugars on BLG variants A and B
Results & Discussion-II

3.2 Effect of heating with sugars on BLG variants A and B

3.2.1 Effect of heating at 50° C and 60° C

Temperature is among the most important factors that affects the Maillard reaction (Morgan et al., 1999). Glycation of BLG variants A and B was thus monitored over a range of temperature in aqueous solution at neutral pH using sugar/protein ratio of 1000:1. Glycoconjugates were prepared by incubating the proteins at 50° C and 60° C with ribose, fructose, glucose or lactose. The characterization of glycoconjugates was made using a number of analytical techniques like CD, fluorescence and PAGE. BLG not subjected to incubation is referred to as ‘native’, while that heated under the conditions used in absence of sugar constituted sugars the ‘control’.

3.2.1.1 Modification of amino groups

The quantity of the modified amino groups and glycation degree were deduced from the OPA assay (Church et al., 1983) (Fig. 3.2.1). The measuring principle is based on the formation of 1-alkylthio-2-alkyl isoindoles generated by the reaction of amino groups with ortho-phthaldialdehyde in the presence of a thiol; the produced compound possesses maximum absorbance at 340 nm. Since no amino groups were modified in the native proteins, all results are reported relative to 100% of amino groups of the native protein.

The BLG contains 16 potential reactive primary amino groups including one α-amino and 15 ε-amino groups of lysine residues (Chevalier et al., 2001). On an average 4.6, 3.3, 3.2 and 2.0 NH₂ groups of BLG A, and 5.6, 4.7, 3.7 and 2.1 NH₂ groups of BLG B were modified in the presence of ribose, fructose, glucose and lactose respectively after three days of incubation at 50° C (Fig. 3.2.1, Table 3.2.1). When BLG was heated with lactose at 60° C for 24 hrs, only 1.3 and 2.7 NH₂ groups were modified in BLG A and B, respectively (Table 3.2.1). Thus, it was observed that the susceptibility of the amino groups of BLG B to reaction with sugars was higher than that of BLG A, both at 50° C and 60° C.

Among the four sugars used, ribose induced the highest degree of modification followed by fructose and glucose, while lactose was least reactive as also reported earlier (Nacka et al., 1998; Chevalier et al., 2001). It is well known that ability of
Fig. 3.2.1: Available amino groups of BLG variants A and B heated at 50° C and 60° C

BLG variants were mixed with the desired amount of sugars (R=Ribose, F=Fructose, G=Glucose, L=Lactose) in 1:1000 ratio and incubated at 50° C and 60° C for various time periods as described under methods. All the amino groups of native proteins were available and considered 100% for calculation of extent of modification. Each bar represents the mean of three experiments carried out in triplicate and SD values have been indicated.

( □ ) BLG A  ( □ ) BLG B
Table 3.2.1 Available amino groups\(^a\) of BLG variants A and B subjected to heating with various sugars at 50\(^\circ\) C and 60\(^\circ\) C

<table>
<thead>
<tr>
<th>Treatment</th>
<th>%Glycation(^b) BLG A</th>
<th>%Glycation(^b) BLG B</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ribose 3 days, 50(^\circ) C</td>
<td>4.6</td>
<td>5.6</td>
</tr>
<tr>
<td>Fructose 3 days, 50(^\circ) C</td>
<td>3.3</td>
<td>4.7</td>
</tr>
<tr>
<td>Glucose 3 days, 50(^\circ) C</td>
<td>3.2</td>
<td>3.7</td>
</tr>
<tr>
<td>Lactose 3 days, 50(^\circ) C</td>
<td>2.0</td>
<td>2.1</td>
</tr>
<tr>
<td>Lactose 24 hrs, 60(^\circ) C</td>
<td>1.3</td>
<td>2.7</td>
</tr>
</tbody>
</table>

\(^a\) Available amino groups were quantified by the method of Church \textit{et al.} (1983) as detailed in methods.

\(^b\) Each value represents the mean of at least three independent experiments performed in duplicate.
reducing sugars to react with proteins increases with rates of their undergoing mutarotation and decreases with the sugar size (Nacka et al., 1998). Shorter the carbon chain of the sugar, greater is the fraction of the molecules that exist in open chain forms and more reactive is the sugar with proteins (Chevalier et al., 2001).

3.2.1.2 Effect on spectral properties

3.2.1.2.1 Near UV-CD

The near-UV CD signal is due to the chirality of the environment of the side chain of aromatic amino acids such as tryptophan, tyrosine and phenylalanine, as well as of disulfide bonds. A characteristic near UV-CD profile was observed in the case of native BLG A (Fig. 3.2.2) and BLG B (Fig. 3.2.3). The two minima observed at about 262 nm and 269 nm in near-UV CD spectrum are due to phenylalanyl residues and the two characteristic deep minima at 286 and 293 nm involve tryptophanyl residues of proteins, the latter in case of BLG, are attributed to Trp19 (Manderson et al., 1999). On heating BLG at 50° C for 3 days, a small alteration in the near UV-CD profile of the two variants was observed (Fig. 3.2.2, 3.2.3). BLG A and B samples heated with glucose (Fig. 3.2.2 D, 3.2.3 D) and lactose (Fig. 3.2.2 E, 3.2.3 E) showed comparable profiles in the near UV region with native and protein heated in the absence of sugar. This suggests that the glycation with the sugars did not modify the environment of Trp19 residue and that only surface lysyl residues of BLG are modified by moderate glycosylation. The CD spectra of BLG modified with ribose (Fig. 3.2.2 B, 3.2.3 B) and fructose (Fig. 3.2.2 C, 3.2.3 C) were not interpretable because of the total loss of information in the near UV region. The profiles obtained could be explained either by a total disappearance of the tertiary structure in the glycated proteins and/or by the possible interference by the chromophores resulting from the Maillard reaction. The complete destruction of tertiary structure may expose lysyl residues buried inside the molecule making them available for reaction with the sugar. The behaviour of the two variants in this study was similar..

CD spectra of BLG molecules exposed to 60° C suggest that, the tertiary structure of BLG heated even without sugar was modified to a small extent and the alteration was enhanced in the presence of lactose (Fig. 3.2.4). Also, the loss in tertiary structure was more prominent in case of BLG B (Fig. 3.2.4 B) than for variant A (Fig. 3.2.4 A), when the proteins were heated in the presence of lactose.
Fig. 3.2.2: Effect of heating with sugars at 50° C on near UV-CD spectrum of BLG variant A

BLG variant A dissolved in 10 mM phosphate buffer, pH 7.0 with 0.02% sodium azide was subjected to glycation at 50° C with various sugars for 3 days. Sugar/protein molar ratio used was 1000:1. BLG A was heated with no sugar (A), ribose (B), fructose (C), glucose (D) and lactose (E).

( ) BLG A-Native

( —— ) BLG A incubated with or without sugars at 50° C
Fig. 3.2.3: Effect of heating with sugars at 50° C on near UV-CD spectrum of BLG variant B

BLG variant B dissolved in 10 mM phosphate buffer, pH 7.0 with 0.02% sodium azide was subjected to glycation at 50° C with various sugars for 3 days. Sugar/protein molar ratio used was 1000:1. BLG B was heated with no sugar (A), ribose (B), fructose (C), glucose (D) and lactose (E).

(---) BLG B-Native
(---) BLG B incubated with or without sugars at 50° C
RESULTS & DISCUSSION-II

Fig. 3.2.4: Effect of heating with sugars at 60° C on near UV-CD spectra of BLG variants A and B

BLG variants A and B dissolved in 10 mM phosphate buffer, pH 7.0 with 0.02% sodium azide were subjected to glycation at 60° C with lactose for 24 hrs. Sugar/protein molar ratio used was 1000:1.

**Panel A:** BLG A (——) Native, (---) Control, (----------) with lactose

**Panel B:** BLG B (——) Native, (---) Control, (----------) with lactose
3.2.1.2.2 Far UV-CD

Far-UV CD signals arise from the peptide bonds absorption and reflect the secondary structure of proteins. BLG shows a negative minimum at 216 nm characteristic of β-sheet proteins (Dong et al., 1996). A slight increase in the intensity of the negative signal was seen in case of BLG A (Fig. 3.2.5 A) and B (Fig. 3.2.6 A) samples heated at 50° C in the absence of sugars but no global secondary structural disorganization was observed, implying that BLG has retained its secondary structure.

Contrary to previous reports (Chevalier et al., 2002), BLG heated with lactose at 60° C showed a clear shifting of negative maxima at 216 nm to 208 nm. This suggests unfolding of β-sheet elements into random coil, both of BLG A (Fig. 3.2.7 A) and B (Fig. 3.2.7 B).

3.2.1.2.3 Intrinsic fluorescence

Trp fluorescence studies of the BLG variants showed maximum emission intensity around 332 nm, representative of a folded conformation and upon heating sugars at 50° C, both the emission maxima and intensity were not altered (Fig. 3.2.8, 3.2.9). However, the intensity of fluorescence was reduced in the presence of sugars without affecting emission maxima and the reduction was proportional to the degree of glycation (table 3.2.1). BLG A glycated with ribose (Fig. 3.2.8 B) and fructose (Fig. 3.2.8 C) showed more decrease in fluorescence intensity than the samples glycated with glucose (Fig. 3.2.8 D) and lactose (Fig. 3.2.8 E). The latter sugars showed profiles comparable to native and control samples (Fig. 3.2.8 A). BLG B on the other hand, showed more marked decrease in fluorescence intensities upon glycation with all the sugars than BLG A but the decrease was most prominent in samples glycated with ribose (Fig. 3.2.9 B) and fructose (Fig. 3.2.9 C). The observed decrease in the intrinsic fluorescence may be attributed to the exposure of tryptophan residues to polar solvent molecules that collide with fluorophores and consume the fluorescence energy.

Both BLG A (Fig. 3.2.10 A) and BLG B (Fig. 3.2.10 B) glycated with lactose at 60° C exhibited a slight red shift in the emission maxima, in addition to the decrease in fluorescence intensity. The result suggests a change in conformation of both BLG variants upon glycation at the temperature used and consequently a changed
Fig. 3.2.5: Effect of heating with sugars at 50° C on far UV-CD spectrum of BLG variant A

BLG variant A dissolved in 10 mM phosphate buffer, pH 7.0 with 0.02% sodium azide was subjected to glycation at 50° C with various sugars for 3 days. Sugar/protein molar ratio used was 1000:1. BLG A was heated with no sugar (A), ribose (B), fructose (C), glucose (D) and lactose (E).

(— — ) BLG A-Native

(---) BLG A incubated with or without sugars at 50° C
**Fig. 3.2.6:** Effect of heating with sugars at 50°C on far UV-CD spectrum of BLG variant B

BLG variant B dissolved in 10 mM phosphate buffer, pH 7.0 with 0.02% sodium azide was subjected to glycation at 50°C with various sugars for 3 days. Sugar/protein molar ratio used was 1000:1. BLG B was heated with no sugar (A), ribose (B), fructose (C), glucose (D) and lactose (E).

(---) BLG B-Native

(----) BLG B incubated with or without sugars at 50°C
Fig. 3.2.7: Effect of heating with sugars at 60°C on far UV-CD spectra of BLG variants A and B

BLG variants A and B dissolved in 10 mM phosphate buffer, pH 7.0 with 0.02% sodium azide were subjected to glycation at 60°C with lactose for 24 hrs. Sugar/protein molar ratio used was 1000:1.

Panel A: BLG A (———) Native, (— — ) Control, (———•) with lactose
Panel B: BLG B (———) Native, (— — ) Control, (———•) with lactose
**Fig. 3.2.8: Effect of heating with sugars at 50° C on intrinsic fluorescence spectrum of BLG variant A**

BLG variant A dissolved in 10 mM phosphate buffer, pH 7.0 with 0.02% sodium azide was subjected to glycation at 50° C with various sugars for 3 days. Sugar/protein molar ratio used was 1000:1. BLG A was heated with no sugar (A), ribose (B), fructose (C), glucose (D) and lactose (E).

( ) BLG A-Native

( - - - ) BLG A incubated with or without sugars at 50° C
Fig. 3.2.9: Effect of heating with sugars at 50° C on intrinsic fluorescence spectrum of BLG variant B

BLG variant B dissolved in 10 mM phosphate buffer, pH 7.0 with 0.02% sodium azide was subjected to glycation at 50° C with various sugars for 3 days. Sugar/protein molar ratio used was 1000:1. BLG B was heated with no sugar (A), ribose (B), fructose (C), glucose (D) and lactose (E).

(—) BLG B-Native

(– – –) BLG B incubated with or without sugars at 50° C
Fig. 3.2.10: Effect of heating with sugars at 60° C on intrinsic fluorescence spectra of BLG variants A and B

BLG variants A and B dissolved in 10 mM phosphate buffer, pH 7.0 with 0.02% sodium azide were subjected to glycation at 60° C with lactose for 24 hrs. Sugar/protein molar ratio used was 1000:1.

Panel A: BLG A (——) Native, (—-—-) Control, (………) with lactose
Panel B: BLG B (——) Native, (—-—-) Control, (………) with lactose
microenvironment of the Trp residues. This result is in good agreement with the near UV-CD data as discussed earlier. As observed from near UV-CD profiles, the decrease in the intrinsic fluorescence intensity was more marked for BLG B samples than for BLG A. It is known that the two amino acid substitutions (Asp64 in A to Gly64 in B and Val118 in A to Ala118 in B), results in destabilization of the core of BLG B molecules (Sawyer and Kontopidis, 2000). This inherent characteristic of the variant B might contribute towards higher thermo-lability and also towards its greater susceptibility towards conformational changes upon attachment of sugar moieties, compared to the variant A.

3.2.1.2.4 ANS fluorescence

The results of ANS fluorescence of native and BLG glycated with lactose at 60° C are shown in figure 3.2.11 A and B for BLG variant A and B, respectively. A marked increase in the ANS fluorescence intensity at 480 nm was observed in control and glycated samples but the increase was more pronounced in the presence of lactose. An increase in the ANS binding sites upon heating of BLG in 20-80° C range has also been shown by Lametti et al. (1995). Such changes indicate that BLG had swollen during the treatment with sugar and produced newly exposed hydrophobic patches on its surface. Also, conformational changes in the protein induced in the presence of sugars as discussed in near UV-CD, far UV-CD and intrinsic fluorescence sections may have contributed to the exposure of new hydrophobic patches on the surface of the protein. The increase in ANS binding was again more pronounced for BLG B than for BLG A.

3.2.1.2.5 New fluorescence

BLG variants A and B glycated with various sugars at 50° C (Fig. 3.2.12, 3.2.13) and 60° C (Fig. 3.2.14 A) exhibited an additional fluorescence peak, which was broad and centered at excitation wavelength of 350 nm and emission wave length of 420 nm compared to native proteins. This additional fluorescence peak may be attributed to the AGE-products (Narayan Murthy and Sun, 2000; Fatima et al., 2008). This fluorescence intensity can be used as a quantitative measure of the content of Maillard products (Miyazawa et al., 1998). At 50° C the observed new fluorescence was more pronounced for ribosylated proteins (Fig. 3.2.12 B, 3.2.13 B), followed by
Fig. 3.2.11: Effect of heating with sugars at 60° C on ANS fluorescence spectra of BLG variants A and B

BLG variants A and B dissolved in 10 mM phosphate buffer, pH 7.0 with 0.02% sodium azide were subjected to glycation at 60° C with lactose for 24 hrs. Sugar/protein molar ratio used was 1000:1.

**Panel A:** BLG A (---) Native, (- - -) Control, (--------) with lactose

**Panel B:** BLG B (---) Native, (- - -) Control, (--------) with lactose
Fig. 3.2.12: Effect of heating with sugars at 50° C on new fluorescence spectrum of BLG variant A.

BLG variant A dissolved in 10 mM phosphate buffer, pH 7.0 with 0.02% sodium azide was subjected to glycation at 50° C with various sugars for 3 days. Sugar/protein molar ratio used was 1000:1. BLG A was heated with no sugar (A), ribose (B), fructose (C), glucose (D) and lactose (E).

( ——— ) BLG A-Native
( ——— ) BLG A incubated with or without sugars at 50° C
Fig. 3.2.13: Effect of heating with sugars at 50° C on new fluorescence spectrum of BLG variant B

BLG variant B dissolved in 10 mM phosphate buffer, pH 7.0 with 0.02% sodium azide was subjected to glycation at 50° C with various sugars for 3 days. Sugar/protein molar ratio used was 1000:1. BLG B was heated with no sugar (A), ribose (B), fructose (C), glucose (D) and lactose (E).

(———) BLG B-Native

( — — — ) BLG B incubated with or without sugars at 50° C
Fig. 3.2.14: Effect of heating with sugars at 60° C on new fluorescence spectra of BLG variants A and B

BLG variants A and B dissolved in 10 mM phosphate buffer, pH 7.0 with 0.02% sodium azide were subjected to glycation at 60° C with lactose for 24 hrs. Sugar/protein molar ratio used was 1000:1.

Panel A: BLG A (---) Native, (-----) Control, (---------) with lactose
Panel B: BLG B (---) Native, (-----) Control, (---------) with lactose
RESULTS & DISCUSSION-II

Fructosylated protein samples (Fig. 3.2.12 C, 3.2.13 C). The intensity of new AGE fluorescence was much lower for glucosylated (Fig. 3.2.12 D, 3.2.13 D) and lactosylated (Fig. 3.2.12 E, 3.2.13 E) protein samples. Thus the new fluorescence detected in glycated samples appears to be related both to the type or extent of Maillard product generated which in turn is related to the nature of sugar (table 3.2.1).

3.2.1.3 Polyacrylamide gel electrophoresis

On reducing SDS-PAGE, bands of BLG A (Fig. 3.2.15 A) and BLG B (Fig. 3.2.15 B) exposed to various sugars at 50°C appear diffused because of possible heterogeneity of molecular mass of glycated proteins as also observed earlier (Morgan et al., 1999; Nacka et al., 1998). The bands appeared more diffused in case of samples incubated with ribose and fructose than those with glucose and lactose. On non-denaturing PAGE, the mobility of the BLG A (Fig. 3.2.15 C) and BLG B (Fig. 3.2.15 D) incubated with sugars towards anode was enhanced and both protein aggregation and band diffusion was evident. As mentioned earlier, a loss in the sharpness of bands may arise because of heterogeneity of molecular mass of proteins as a result of reaction with sugars. The increased mobility of glycated protein towards the anode has been attributed to a decrease in the positive charge resulting from the attachment of sugar residues to the amino groups of the proteins (Handa and Kuroda, 1999). Also, the increase in the net negative charge of glycated proteins may occur from β-elimination and enolization of the α-hydroxy carbonyl group of the sugar moiety of the Amadori products under the alkaline conditions of PAGE, to form an enolate anion (Miksik and Deyl, 1997).

On reducing SDS-PAGE (Fig. 3.2.16 A), BLG A and B samples heated with lactose at 60°C showed migration comparable to the native protein and that heated in the absence of sugar. However, the increase in molecular mass of the BLG variants upon lactose attachment could not be discerned from the electrophoretic mobility under reducing conditions. On non-reducing SDS-PAGE, bands corresponding mainly to dimers and trimers appeared in samples of both BLG variants heated in absence or presence of lactose (Fig. 3.2.16 B). At the same time, aggregates that were too large to enter the gels were seen especially in the BLG B samples. Oligomerisation of BLG molecule upon heating is well known and occurs mainly by disulfide exchange in which the free Cys121 plays an important role (Iametti et al., 1995). Maillard reaction
Fig.3.2.15: Polyacrylamide gel electrophoresis of the BLG variants heated with various sugars at 50° C.

Gel electrophoresis of BLG variants heated in the absence and presence of ribose, fructose, glucose and lactose was performed at 50° C for 3 days at pH 7.0 on a 15% gel. The gels were run according to the procedure of Laemmli (1970) and staining was done with CBB R-250. 20 µg or 35 µg protein was loaded in each well.

Panel A & B: Reducing SDS-PAGE of BLG A and B, respectively.
Panel C & D: Non-denaturing PAGE BLG A and B, respectively.
Fig. 3.2.16: Polyacrylamide gel electrophoresis of the BLG variants heated with sugars at 60° C.

Gel electrophoresis of BLG variants heated in the absence and presence of lactose at 60° C for 24 hrs at pH 7.0 was performed on 15% gel. Lanes 1-6 contain native BLG A, control BLG A, BLG A heated with lactose, native BLG B, control BLG B and BLG B heated with lactose, respectively. Lane M contains molecular weight markers as described previously. 20, 30 or 10 μg protein was loaded in each well.

Panel A, B, C: Reducing SDS-PAGE, non-reducing SDS-PAGE and non-denaturing PAGE
is also known to cause protein cross-links (Miller and Gerrard, 2005). Figure 3.2.16 C shows that migration behaviour under non-denaturing conditions of BLG variants heated with sugars is comparable with the controls.

Gel electrophoresis of glycated protein samples performed either in the presence or in the absence of SDS provides information about cross-linking/polymerization of proteins during incubation. Cross-linked proteins are larger in dimension and hence, have lower electrophoretic mobility than non-cross-linked protein species (Morgan et al., 1999). In the presence of SDS, the average molecular weight of cross-linked/oligomerized protein species can be estimated while in the absence of SDS, PAGE can be used to reveal changes in the charge properties of the glycated proteins (Miksik and Deyl, 1997).

3.2.2 Effect of exposure of BLG variants A and B to high temperatures in absence or presence of some sugars

3.2.2.1 Migration behaviour in SDS-polyacrylamide gels

In the first set of experiments, BLG variants A and B were heated either at 65°C or 85°C for 20 min in absence or presence of the highly reactive aldopentose ribose and the less reactive disaccharide occurring in milk lactose (Chevalier et al., 2001). SDS-PAGE profiles of the control and heated samples are shown in figure 3.2.17.

Non-reducing SDS-PAGE of BLG A (Fig. 3.2.17 A) and B (Fig. 3.2.17 B) heated at 65°C for 20 minutes revealed moderate aggregation, as evident from the appearance of some higher molecular weight bands. Heating of the samples to 85°C however, resulted in a striking increase in aggregation indicated by the appearance of increased number of such bands corresponding to dimers, trimers and higher oligomers. Clearly the oligomerization/aggregation was more marked in case of BLG variant B (Fig. 3.2.17 B) as evident from the larger number of faint bands occupying nearly all the area below the top of gel. The observed aggregation of the BLG variants in response to heating in presence or absence of sugar was primarily disulfide mediated, since SDS-PAGE performed in presence of β-mercaptoethanol gave a single prominent band corresponding to the monomer (Fig. 3.2.17 C, 3.2.17 D). Faint bands corresponding to higher molecular weight BLG molecules are evident in all lanes including those of native and control samples probably represent small fractions of the variants recalcitrant to the thiol-reductant. However, in case of the variants A and B
Fig. 3.2.17: Polyacrylamide gel electrophoresis of BLG variants A and B heated with sugars at 65° C and 85° C.

SDS-PAGE of BLG A and B heated with and without lactose and ribose at 65° C and 85° C for 20 min at pH 7.0 was performed as detailed earlier using 15% gels. 35 μgm of protein was loaded in each well.

Panel A & B: Non-reducing SDS-PAGE of BLG A and B, respectively.
Panel C & D: Reducing SDS-PAGE of BLG A and B, respectively.
heated in presence of ribose, significant fraction of the dimer remained in gels run in presence of β-mercaptoethanol, suggesting an association of the monomers not mediated by the disulfides. That the high reactivity of sugars such as ribose with proteins leads to the formation of AGEs, some of which induce protein cross-links is well recognized (Miller and Gerrard, 2005). Heating of the BLG variants apparently leads mainly to the formation of disulfide stabilized aggregates. The free thiol (Cys121) group of BLG is known to participate in the aggregation of the protein, especially at high temperatures (Qin et al., 1998).

The next set of experiments was carried out with the more reactive ribose. Extension of incubation time of BLG with the sugar at 65° C or 85° C did not result in additional qualitative alteration in the electrophoretic behaviour of either variant of BLG. Figure 3.2.18 and 3.2.19 show that extension in incubation time at 65° C or 85° C of the BLG variants A or B in presence or absence of ribose lead to an enhancement in the formation of dimers and to small extent the higher oligomers. The relatively higher susceptibility of the variant B to the heat-induced aggregation is also supported from the study. Although, no marked increase was observed in the formation of non-disulfide linked dimers in case of either of the variants at 65° C (Fig. 3.2.18 C, 3.2.18 D), at 85° C a remarkable increase in non-disulfide linked dimers was observed in case of both the variant and A (Fig. 3.2.19 C) and B (Fig. 3.2.19 D). The increase was somewhat more marked in case of the latter. The Rayleigh scattering data (Fig. 3.2.20) of the samples heated at 85° C provide more convincing evidence of the higher susceptibility of the variant B towards aggregation in the absence and presence of sugars. As shown the FI measured at 350 nm increased when either of the BLG variants was heated but the increase was more marked when lactose and more significantly ribose were present at the time of heating. In these experiments also the increase was more marked in case of the variant B.

Figure 3.2.21 depicts the extent of modification of amino groups of BLG variants heated in presence of the sugars, as determined using OPA assay (Church et al., 1983). Relatively small fraction (approximately 1.0) of the total amino groups in BLG were modified when the proteins were treated with lactose, while ribose treatment at 85° C resulted in the modification of about four amino groups (table 3.2.2). The result is in accordance to earlier reports on the reactivities of the sugars used (Chevalier et al., 2001). There was no significant difference between the variants A and B in
Fig. 3.2.18: Polyacrylamide gel electrophoresis of BLG variants A and B heated with sugars at 65°C.

SDS-PAGE (12%) of BLG A and B heated with and without ribose at 65°C for 20 min and 1 hr at pH 7.0. 35 μg of protein was loaded in each well.

Panel A & B: Non-reducing SDS-PAGE of BLG A and B, respectively.
Panel C & D: Reducing SDS-PAGE of BLG A and B, respectively.
Fig. 3.2.19: Polyacrylamide gel electrophoresis of BLG variants A and B heated with sugars at 85°C.

SDS-PAGE (12%) of BLG A & B heated with and without ribose at 85°C for 20 min and 1 hr at pH 7.0. 35 μg of protein was loaded in each well.

Panel A & B: Non-reducing SDS-PAGE of BLG A and B, respectively.
Panel C & D: Reducing SDS-PAGE of BLG A and B, respectively.
Fig. 3.2.20: Effect of heating with sugars on RLS of the BLG variants

Rayleigh scattering data of BLG variants heated with and without sugars at 85° C for 20 min is shown.

(■) BLG A  (▲) BLG B
Table 3.2.2 Available amino groups\(^\text{a}\) of BLG variants A and B subjected to heating with various sugars at 65\(^\circ\) C and 85\(^\circ\) C

<table>
<thead>
<tr>
<th>Treatment</th>
<th>%\text{Glycation}(^b)</th>
<th>%\text{Glycation}(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BLG A</td>
<td>BLG B</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Lactose 20 min, 65(^\circ) C</td>
<td>6.3</td>
<td>7.1</td>
</tr>
<tr>
<td>Ribose 20 min, 65(^\circ) C</td>
<td>8.6</td>
<td>9.3</td>
</tr>
<tr>
<td>Lactose 20 min, 85(^\circ) C</td>
<td>7.6</td>
<td>8.9</td>
</tr>
<tr>
<td>Ribose 20 min, 85(^\circ) C</td>
<td>13.0</td>
<td>14.7</td>
</tr>
<tr>
<td>Ribose 1 hr, 85(^\circ) C</td>
<td>22.4</td>
<td>29.1</td>
</tr>
</tbody>
</table>

\(^\text{a}\)Available amino groups were quantified by the method of Church \textit{et al.} (1983) as detailed in methods.

\(^\text{b}\)Each value represents the mean of at least three independent experiments performed in duplicate.
Fig. 3.2.21: **Available amino groups of BLG variants A and B heated at 65° C and 85° C**

Protein solutions were mixed with sugars (R=Ribose, L=Lactose) in molar ratio 1:1000 and incubated at different temperatures (65° C, 85° C) for various time periods as indicated. All the amino groups of native proteins were available and considered 100% for calculation of extent of modification. Each bar represents the mean of three experiments carried out in triplicate and SD values have been indicated.

(■) BLG A  (■■) BLG B
susceptibility to modification by sugars although the latter appeared modified to a slightly higher extent.

Hence, it can be said that appearance of high molecular weight species (dimers, trimers, tetramers etc.) was more pronounced at 85° C than at 65° C and for the variant B compared to variant A at both temperatures. This corresponds well with the Rayleigh scattering data as well as with the slightly higher extent of glycation observed in variant B from the OPA assay. The variation between amino acid sequence between the variants A and B is restricted to position 64 (Asp64-Gly) in a mobile surface loop and position 118 (Val118-Ala) in the hydrophobic core. The observed difference between the thermal behaviour between the variants may thus result from higher destabilisation of the core of the variant B relative to that of A because of the cavity formed by the loss of the two methyl groups in the later and hence to lowered hydrophobicity (Sawyer and Kontopidis, 2000).

3.2.2.2 Effects on Conformation

Conformational alterations in the BLG variants subjected to high temperatures in presence or absence of lactose and ribose were monitored using far UV-CD and intrinsic fluorescence. BLG is a predominantly β-sheet protein (Dong et al., 1996) and has a characteristic negative minima at 216 nm. It can be seen from far UV-CD data of variant A (Fig 3.2.22 A) and variant B (Fig 3.2.22 B) that heating of either with or without sugars resulted in a moderate enhancement of CD signals at ~208 nm suggesting a destabilising effect on the protein’s secondary structure and transition from the β-sheet structure to random coil (Prabhakaran and Damodaran, 1997) by sugars in the order:

Heated Control< BLG Lactose< BLG Ribose.

Heating of the BLG A and B in the presence of sugars also brought about alterations in the intrinsic fluorescence (Fig. 3.2.22 C, 3.2.22 D). A small red shift (~2-3 nm) accompanied by increase in fluorescence intensity was observed when the variants heated without sugars were excited at 280 nm. The increase in FI suggests exposure of Trp to aqueous environment presumably resulting from unfolding of the protein (lametti et al., 1998). BLG heated in presence of sugars shows some quenching of Trp fluorescence as compared to the control heated without sugar and the effect was more pronounced with ribose (curve 4) as compared to lactose (curve 3). It has been
Fig. 3.2.22: Effect of heating with sugars at 85° C on far UV-CD and intrinsic fluorescence spectra of the BLG variants

Spectroscopic data of BLG variants heated with and without sugars (molar ratio 1:1000) at 85° C for 20 min is shown. Curves 1, 2, 3 and 4 correspond to Native BLG, Heated BLG control, BLG heated with lactose and BLG heated with ribose, respectively.

Panel A & B: Far UV-CD spectra of BLG A and B, respectively.
Panel C & D: Intrinsic fluorescence spectra of BLG A and B, respectively.
reported that interaction of Trp19 with quenching side-chain groups such as Arg124 (Brownlow et al., 1997) in the protein are removed upon heat treatment resulting in increased fluorescence (Iametti et al., 1998). It is likely that in the BLG molecules heated in presence of sugars the disruption of such interaction is less marked due to additional conformational alterations.

### 3.2.2.3 Susceptibility to Pepsinolysis

The digestion of milk proteins is initiated in the stomach by pepsin, continues in the intestine and the digestion products are absorbed from the intestinal (Kitabatake and Kinekawa, 1998). Among milk proteins BLG is highly nutritious and is often used as a base for infant formulae, in spite of its known resistance to pepsinolysis (Schmidt and Van Marwisk, 1993; Kitabatake and Kinekawa, 1998). The effect of heat treatment and glycation on susceptibility of the BLG variants A and B to porcine pepsin was examined both by SDS-PAGE and RP-HPLC. BLG variants containing lactose or ribose in molar ratio 1:1000 were exposed to 85°C for 20 min and their susceptibility to cleavage with porcine pepsin was investigated.

Figure 3.2.23 shows the SDS-PAGE profile of variants A (Fig. 3.2.23 A) and variant B (Fig. 3.2.23 B) exposed to high temperature in presence of lactose. Unheated BLGs (lane 2) appeared recalcitrant to pepsinolysis. Heating with or in absence of sugars rendered the BLG variants susceptible to proteolysis which could be monitored by SDS-PAGE. BLG samples heated in presence of ribose became even more susceptible to hydrolysis by pepsin. The relative rates of pepsin-induced hydrolysis of the 18 kDa band corresponding to BLG was observed to be:

Heated control < BLG-Lactose < BLG-Ribose

No distinct low molecular weight peptide could be visualised in the stained gels presumably due to the formation of small peptides with poor ability to bind Coomassie brilliant blue R-250.

Additional support for the observations came from RP-HPLC. Figure 3.2.23 C depicts the extent of hydrolysis of BLG A and B after 3 hrs of pepsin treatment. Only about 12% and 11% of native BLG A and B, respectively were hydrolysed showing high resistance of the proteins to pepsin. The susceptibility to proteolysis increased remarkably when heating was carried out especially in the presence of the sugars. While about 25% of BLG A was hydrolysed on heating in the absence of sugar for 20
Fig. 3.2.23: Effect of heating the BLG variants with sugars on susceptibility to pepsinolysis

BLG A & B incubated with lactose and ribose at 85° C for 20 min were subjected to pepsinolysis as described in methods.

**Panel A & B:** Reducing SDS-PAGE (15%) of BLG A and B, respectively. Lane M shows molecular weight markers. ~15 μg each of native untreated protein, pepsin treated native protein, pepsin treated heated protein, pepsin treated BLG-L and pepsin treated BLG-R was loaded in each well.

**Panel C:** Peptic hydrolysates of native and enzyme treated BLG A & B were separated by RP-HPLC as described in methods. The relative peak areas are plotted against respective samples. Each bar represents the mean of three experiments carried out in triplicate.

(□) BLG A  (■) BLG B
minutes, that heated in presence of lactose and ribose was hydrolysed to ~50% and 70%, respectively. Susceptibility to pepsinolysis also increased in a similar fashion and the corresponding values for variant B were ~27%, ~53% and ~74%, respectively. These results suggest that the effect of heat treatment at 85° C produce nearly parallel effects on the BLG variants although the later seems to turn slightly susceptible. Native BLG is known to have a highly stable conformation and most of the cleavage sites of the pepsin are buried inside the β-barrel at low pH (Guo et al., 1995) rendering the protein highly resistant towards peptic hydrolysis. Exposure to high temperature evidently induces conformational changes in the protein facilitating pepsinolysis. Further, BLG aliquots heated with sugars were more susceptible than the samples heated in absence of the sugars suggesting the role of reaction with the sugars in causing unfolding of protein. Also, the results show that ribose modified BLG is somewhat more susceptible than that modified with lactose. Pepsinolysis experiments also suggest higher susceptibility of BLG B exposed to high temperature in presence of sugars presumably due to its higher susceptibility to thermal denaturation and glycation (Sawyer and Kontopidis, 2000).

3.2.3 Effects of high temperature on thiol modified BLG Variants A and B

3.2.3.1. Effect of thiol modification

The extraordinary stability of BLG at low pH has been explained by the strong stabilising action of the two disulfide bonds present in the molecule which help in preserving several features of the secondary structure at physiological pH (Molinari et al., 1996). The free thiol groups of Cys121 in each monomer has been shown to be involved in intramolecular and intermolecular disulfide interchange especially at elevated temperature (Griffin et al., 1993; Roefs and DeKruif, 1994) or high hydrostatic pressures (Tanaka et al., 1996; Lametti et al., 1997; Funtenberger et al., 1997).

The behaviour of the BLG variants A and B, with chemically modified free sulfhydryls and subjected to heat treatment in absence or presence of sugars was also investigated. Sulfhydryl modified BLG was prepared by trapping the lone free thiol group in the transiently dissociated monomers by reaction with IAA in the presence of 2M GnHCl at pH 6.8 (Lametti et al., 1998). Iodoacetamide is widely used to block free cysteine residues of proteins during characterization and peptide mapping. Alkylation with iodoacetamide results in the covalent addition of a carbamidomethyl group (57.07
Da) on reduced cysteine residues and prevents its participation in disulfide bond formation. If iodoacetamide is present in limiting quantities and a slightly alkaline pH, as has been used in this study, cysteine modification is the exclusive reaction (Product instructions, Thermo Fisher Scientific Inc. USA, 2009).

3.2.3.1 Spectral Properties

The extent of modification of free thiol groups was determined using the assay employing the Ellman’s reagent. As shown in table 3.2.3, near complete modification of the free sulfhydryl groups was achieved under the condition employed (Ellman, 1959). The structural features of the blocked monomers of both variants were compared with those of the respective native proteins at pH 7.0 (Fig. 3.2.24).

The characteristic features of the far UV-CD spectrum of the native BLG that exists as a dimer (Sawyer and Kontopidis, 2000) were slightly modified both in the thiol modified BLG A (Fig 3.2.24 A) and BLG B (Fig. 3.2.24 B). The observed blue shift of the negative peak at 216 nm (Dong et al., 1996) indicates some conversion of β-sheet and α-helix to aperiodic structures (Prabhakaran and Damodaran, 1997). Further details of the nature of alterations induced by sulfhydryl modification of BLG came from intrinsic fluorescence. The tryptophan fluorescence of the BLG variants with blocked sulfhydryl shows higher emission intensity than the respective native dimeric BLG A (Fig. 3.2.24 C) and BLG B (Fig. 3.2.24 D), in agreement with the results of lametti et al. (1998). This indicated interference in the formation of dimers in the thiol modified variants.

3.2.3.1.2 Aggregation Characteristics

A small fraction of the thiol modified variants A and B migrated as a dimer even when the samples were not exposed to high temperature (Fig. 3.2.25, 3.2.26). The dimers appeared disulfide linked since SDS-PAGE in presence of the β-mercaptoethanol resulted in their near disappearance from the gels. It is likely that a small fraction of Cys residues remaining after the treatment of BLG with IAA (table 3.2.3) cause the re-association of few BLG monomers by disulfides. Also, a decrease in sulfhydryl content, which would prevent the disulfide exchanges and formation of extensive intermolecular disulfide linkages, may have actually increased the molecular flexibility of the BLG molecules (Xiong, et al., 1993). The latter would have produced
Table 3.2.3  Free sulphhydril groups<sup>a</sup> of native and IAA treated BLG A and B

<table>
<thead>
<tr>
<th>Sample</th>
<th>nmoles of SH groups&lt;sup&gt;b&lt;/sup&gt;</th>
<th>% modification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native BLG A</td>
<td>5.48</td>
<td>0</td>
</tr>
<tr>
<td>IAA treated BLG A</td>
<td>0.288</td>
<td>94.7</td>
</tr>
<tr>
<td>Native BLG B</td>
<td>5.32</td>
<td>0</td>
</tr>
<tr>
<td>IAA treated BLG B</td>
<td>0.2</td>
<td>96.2</td>
</tr>
</tbody>
</table>

<sup>a</sup>Free groups were quantified by the method of Ellman <i>et al.</i> (1959) as detailed in methods.

<sup>b</sup>Each value represents the mean of at least three independent experiments performed in duplicate.
Fig. 3.2.24: Effect of thiol modification on far UV-CD and intrinsic fluorescence of BLG A and BLG B

BLG A & B in 50 mM phosphate buffer, pH 6.8 were treated with iodoacetamide as described in the methods.

Panel A & B: Far UV-CD spectra of BLG A and B, respectively.
Panel C & D: Intrinsic fluorescence spectra of BLG A and B, respectively.

(—) BLG A  (—-—) thiol blocked BLG A
(—) BLG B  (—-—) thiol blocked BLG B
enhanced molecular interactions via some nonspecific short-range bonding such as hydrophobic and van der Waals bonds leading to the formation of small amounts of disulfide linked dimers.

3.2.3.2 Effect of heating on thiol modified BLG variants A and B

3.2.3.2.1 Migration Behaviour in SDS-Polyacrylamide gels

Heating of the thiol modified BLG A (Fig. 3.2.25 A, 3.2.26 A) and B (Fig. 3.2.25 B, 3.2.26 B) with or in absence of lactose or ribose resulted in very little formation of additional dimers or higher oligomers as compared to the native variants (Fig. 3.2.19). However while the intensity of the dimer bands of unheated thiol modified variant A or that heated in absence of the sugar decreased remarkably on inclusion of the thiol-reductant during electrophoresis, greater fraction of the dimer remained in samples heated in presence of the sugars (Fig. 3.2.25 C, 3.2.26 C). This suggests that sugars can induce higher non-disulfide mediated aggregation also in the thiol modified protein although to a very small extent. Almost similar observations were made with the thiol modified BLG variant B except that some additional faint bands appeared above the dimer band and more marked staining on the top of the gel was evident both in the samples heated with or in absence of the sugar (Fig. 3.2.25 B, 3.2.26 B). The dimer band appeared sharper indicating lower heterogeneity and when the sample was subjected to electrophoresis in presence of the thiol-reductant the intensity of the band corresponding to the dimer decreased remarkably in all samples except in that heated with ribose (Fig. 3.2.25 B, D; Fig. 3.2.26 B, D). Fewer amino groups of both the thiol modified BLG variants were modified when they were heated with sugar as compared to those in the respective unmodified protein (Fig. 3.2.27). Treatment of the variants A and B with IAA resulted in a small decrease in the number of amino groups (table 3.2.4) probably because of the ability of the excess reagent to alkylating amines to some extent (lysine, N-termini) (Product instructions, Thermo Fisher Scientific Inc. USA, 2009). The observed lower modification of amino groups of the thiol modified BLG incubated with sugars may be the result of the masking effect of some alkylation products on the potential sugar glycation sites. Alternatively, the higher resistance to unfolding of the thiol-modified variants may have restricted the access of the glycation sites and hence lower amino group modification.
Fig. 3.2.25: Polyacrylamide gel electrophoresis of thiol modified BLG variants heated with sugars at 85° C for 20 min.

SDS-PAGE (12%) of thiol modified BLG A and B heated with and without lactose and ribose (molar ratio 1:1000) at 85° C for 20 min at pH 7.0. The thiol blocked proteins are referred to as Cys121 blc BLG A and B. ~35 μgm of protein was loaded in each well.

Panel A & B: Non-reducing SDS-PAGE of thiol modified BLG A and B, respectively.

Panel C & D: Reducing SDS-PAGE of thiol modified BLG A and B, respectively.
Fig. 3.2.26: Polyacrylamide gel electrophoresis of thiol modified BLG variants heated with sugars at 85° C for 1 hr.

SDS-PAGE (12%) of thiol modified BLG A and B heated with and without lactose and ribose (molar ratio 1:1000) at 85° C for 1 hr at pH 7.0. The thiol blocked proteins are referred to as Cys121 blc BLG A and B. ~35 μgm of protein was loaded in each well.

Panel A & B: Non-reducing SDS-PAGE of thiol modified BLG A and B, respectively.
Panel C & D: Reducing SDS-PAGE of thiol modified BLG A and B, respectively.
Fig. 3.2.27: Available amino groups of thiol modified BLG variants A and B heated at 85° C

Thiol modified protein solutions were mixed with sugars (R=Ribose, L=Lactose) in molar ratio 1:1000 and incubated at 85° C for various time periods as indicated. All the amino groups of native proteins were available and considered 100% for calculation of extent of modification. Each bar represents the mean of three experiments carried out in triplicate and SD values have been indicated. The thiol modified BLG A and B are referred to as Cys121 blc A and B.

(▲) BLG A  (▲) BLG B
Native BLG

Cys121 ble BLG unheated

Cys121 ble BLG-L 20 min, 85° C

Cys121 ble BLG-L 1 hr, 85° C

Cys121 ble BLG-R 20 min, 85° C

Cys121 ble BLG-R 1 hr, 85° C
Table 3.2.4 Available amino groups\textsuperscript{a} of thiol modified BLG variants A and B subjected to heating with various sugars at 85° C

<table>
<thead>
<tr>
<th>Treatment</th>
<th>%\textsuperscript{b}Glycation\textsuperscript{b} BLG A</th>
<th>%\textsuperscript{b}Glycation\textsuperscript{b} BLG B</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>6.2</td>
<td>6.2</td>
</tr>
<tr>
<td>Lactose 20 min</td>
<td>7.0</td>
<td>7.5</td>
</tr>
<tr>
<td>Lactose 1 hr</td>
<td>9.3</td>
<td>12.5</td>
</tr>
<tr>
<td>Ribose 20 min</td>
<td>13.1</td>
<td>14.0</td>
</tr>
<tr>
<td>Ribose 1 hr</td>
<td>17.5</td>
<td>18.8</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Available amino groups were quantified by the method of Church et al. (1983) as detailed in methods.

\textsuperscript{b}Each value represents the mean of at least three independent experiments performed in duplicate.
3.2.3.2 Spectral Properties

Far UV-CD data of the thiol-modified variant A and B show a very small loss of secondary structure upon heating in absence or presence of sugars (Fig. 3.2.28 A, B, C, D). Only a small enhancement of CD signals at ~208 nm was observed suggesting a minor transition from β-sheet structure to random coil. The alterations were somewhat more marked, especially in case of the variant B, when the samples were heated in the presence of the sugars. Heating of thiol-modified BLG A and B also resulted in a marked increase in the intrinsic fluorescence (Fig. 3.2.29 A, B, C, D) when excited at 280 nm also suggesting greater exposure of Trp to aqueous environment, presumably due to protein unfolding. Inclusion of lactose or ribose during heating however resulted in significant quenching of Trp fluorescence (curves 3 & 4). As explained earlier, the Maillard reaction of the sugars with Lys residues of the protein might be responsible for greater interaction of Trp19 with quenching side-chains groups such as Arg124 (Brownlow et al., 1997) in the protein resulting in decreased fluorescence.

The genetic variants of milk proteins have recently been of great interest in the dairy industry (Celik, 2003). There are several functional consequences of the amino acid substitutions in variant A and B. The loss of two methyl groups accompanying the Val118 in A to Ala in B occurring in the most rigid part of the protein structure, coupled with the inability of the surrounding structure to adjust to fill this void, results in the decreased degree of internal hydrophobic interactions for BLG B compared to BLG A. Moreover, the extent of destabilization is greatest when substitution occurs in the most rigid part of the structure. According to the X-ray studies by Qin et al. (1999), five hydrophobic contacts are subsequently lost in variant B. Thus, the decreased thermal stability of BLG B at high temperatures and low protein concentrations compared to BLG A arises from the reduced hydrophobic contacts that result from the substitutions. X-ray data shows that in variant B there occurs greater mobility of the region around Cys121 (Qin et al., 1999), indicating greater transient accessibility of this residue and its subsequent greater reactivity in variant B. This together with the lower charge density of BLG B species (Meza-Nieto et al., 2007), compared with BLG A resulting in diminished electrostatic repulsion among former’s molecules, can be accounted for the greater heat induced aggregation tendency of the variant B compared to A. It has been earlier observed that in studies concerning the effect of fortifying reconstituted skim milk with increasing levels of BLG variants A and B and A-B
Effect of thiol modification on far UV-CD of the BLG variants heated with sugars at 85° C.

The thiol blocked BLG variants A & B were subjected to heat treatment at 85° C with or without lactose and ribose in molar ratio 1:1000. Curves 1, 2, 3 and 4 correspond to unheated Cys121 blc BLG, Heated Cys121 blc BLG control, Cys121 blc BLG heated with lactose and Cys121 blc BLG heated with ribose, respectively.

Panel A & C: Far UV-CD spectra of BLG A incubated at 85° C for 20 min and 1 hr, respectively.

Panel B & D: Far UV-CD spectra of BLG B incubated at 85° C for 20 min and 1 hr, respectively.
Fig. 3.2.29: Effect of thiol modification on intrinsic fluorescence of the BLG variants heated with sugars at 85° C.

The thiol blocked BLG variants A & B, were subjected to heat treatment at 85° C with or without lactose and ribose in molar ratio 1:1000. Curves 1, 2, 3 and 4 correspond to unheated Cys121 blc BLG, Heated Cys121 blc BLG control, Cys121 blc BLG heated with lactose and Cys121 blc BLG heated with ribose, respectively.

**Panel A & C:** Intrinsic fluorescence spectra of BLG A incubated at 85° C for 20 min and 1 hr, respectively.

**Panel B & D:** Intrinsic fluorescence spectra of BLG B incubated at 85° C for 20 min and 1 hr, respectively.
mixtures on rennet-induced gelation, BLG B contributed more to the mechanical strength of the gels formed due to formation of cross-links and aggregates with other whey proteins and rennet hydrolysis products.

Hence it can be concluded from this study that in BLG A and B the extent of structural modification induced is related to the degree of glycation, which in turn is related to the reactivity of the sugar used, and to the temperature of incubation. Higher the reactivity of the sugar, higher was the denaturation observed upon glycation. Depending on the reactivity of the sugar, the glycated population shows smaller or greater heterogeneity in molecular masses. Glycosylation of BLG variants at a moderate temperature of 50°C, in the case of glucose and lactose, does not change significantly the secondary and the tertiary structure of the proteins suggesting that only surface lysyl residues of BLG are modified by moderate glycosylation. In contrast, glycosylated BLG derivatives characterized by higher substitution, as in the case of ribose and fructose, reveal complete destruction of tertiary structure, while retaining the native secondary structure. Glycation of BLG variants at 60°C even with a lesser reactive sugar like lactose induced marked loss of native secondary and tertiary structure of the protein accompanied by an increase in hydrophobicity. Although through secondary structure analysis, the behaviour of the two variants upon glycation was found to be comparable, from near UV-CD, intrinsic and ANS fluorescence data it was observed that the environment of Trp19 was modified to a greater extent in case of BLG B upon incubation with sugars, both at 50°C and 60°C. Also, through non-reducing PAGE it was shown that the variant B was more prone to polymerization than variant A upon glycation at 60°C. Additionally, this work showed the difficulty to interpret some of the structural analysis of glycated samples by spectral methods, especially with more reactive sugars like ribose because of the heterogeneity of the Maillard products obtained and presumably because of formation of powerful chromophores during the Maillard reactions.

The heat treatment of BLG variants at temperatures as high as 85°C was found to induce conformational changes in the protein together with increased aggregation under the conditions used. The aggregation is enhanced in the presence of sugars and is related to the reactivity of sugar and extent of glycation. Among the variants BLG B tends to aggregate to a greater extent than BLG A confirming its higher susceptibility towards heat denaturation. The study of BLG hydrolysis by pepsin shows that heating
of milk at high temperatures in the presence of sugars enhances the digestibility of both BLG variants. This may facilitate the absorption rate of heated protein. Also, the intensity of denaturation corresponding to percentage of hydrolysed protein is directly related to glycation degree. The susceptibility of variant B towards pepsinolysis after heat treatment was found to be slightly higher than that of the variant A, possibly because of former’s greater susceptibility towards heat denaturation. Treatment of BLG variants with thiol modifying reagent produces moderate conformational changes in the proteins. Thiol modified BLG variant showed lower susceptibility to glycation and far lower heat-induced aggregation as compared to the unmodified proteins. The aggregation behaviour of both the variants was also comparable as evident from non-reducing SDS-PAGE, although BLG B also appeared more susceptible in this regard. The glycation of BLG was affected probably by the conformational changes induced by the thiol-modifying reagent as well as the presence of covalently attached alkylating groups on the BLG molecules. Heating of the thiol modified BLG variants induced some conformational changes in the protein as monitored by CD and fluorescence spectroscopy but these were of lower magnitude compared to those observed with variants with unmodified thiol group. The alterations were enhanced in the presence of sugars.

Numerous attempts have been made to improve the functional properties of whey proteins through physical, chemical and/or enzymatic treatments (Kehoe et al., 2007). However, most of the methods utilize toxic chemical leading to products like ester and phosphoric acid (Kehoe et al., 2007) for conjugation with proteins like BLG and are not permitted for potential industrial applications. Controlled Maillard reaction can thus be a good procedure for protein processing in food industry, which allows the protein to retain its native conformation.
Acetonitrile-induced unfolding of bovine beta-lactoglobulins
3.3 Acetonitrile-induced unfolding of bovine beta-lactoglobulins

The conformational change of BLG variants A and B as a function of varying concentrations of acetonitrile (ACN) was monitored at pH 2.0, 7.0 and 9.0. The conformational changes were followed using various spectroscopic probes including Rayleigh Light Scattering, intrinsic tryptophan fluorescence, far-UV CD, near-UV CD and ANS fluorescence. pH values of 2.0, 7.0 and 9.0 were chosen to study the effect of increasing concentrations of ACN (0-80%) since it is known that at pH 2.0 and pH 9.0 both of the BLG variants exist as monomers while they exist in dimeric form at pH 7.0 (Sawyer and Kontopidis, 2000). These conditions provide a range of pH to study the effect of ACN on the conformation of BLG variants both in monomeric or in dimeric form.

3.3.1 Rayleigh Light Scattering (RLS)

In order to study the aggregation behaviour of BLG variants induced by ACN, light scattering measurements were made in the presence of increasing concentrations of acetonitrile. Figures 3.3.1 A and 3.3.1 B show the ACN-induced aggregation profile of BLG variants A and B, respectively.

The RLS data showed that BLG variants A and B exhibit comparable aggregation behaviour both in monomeric form (pH 2.0, pH 9.0) as well as in dimeric form (pH 7.0). At pH 2.0 (Fig. 3.3.1 A, 3.3.1 B) increase in concentration of ACN up to 70% (v/v) caused no remarkable aggregation but at 80% (v/v) ACN there was an abrupt rise in fluorescence intensity both for variants A and B. At pH 7.0 both variants exhibited aggregation beyond 50% (v/v) concentration of ACN. At pH 9.0, BLG aggregation was evident above 60% ACN (v/v). ACN-induced aggregation was thus more pronounced at pH 7.0, fluorescence intensity (FI) at 80% (v/v) ACN observed at pH 7.0 is nearly 3 times greater than the maximum FI observed at pH 2.0 or 9.0 at the same ACN concentration. Figures 3.3.1 A and B also provides a threshold acetonitrile concentration to study the protein aggregates formed under the conditions and the conformational conversion of monomeric and dimeric state populated under the same conditions before the aggregates form.
Fig. 3.3.1  Effects of increasing concentration of ACN on aggregation of BLG variants A and B.

Fluorescence measurements were made at 350 nm after exciting the samples at 350 nm. Concentration of BLG A (A) and B (B) used was 10 μM and the measurements were made at (♦) pH 2.0, (■) 7.0 and (△) 9.0.
The graph shows the fluorescence intensity (FI) at 350 nm (A.U.) as a function of the percentage of acetonitrile (%ACN) (v/v). Two separate plots are shown:

**Plot A:**
- Data points are represented by squares and diamonds.
- The x-axis represents %ACN (v/v) ranging from 0 to 100.
- The y-axis represents the range from 0 to 6000.

**Plot B:**
- Data points are represented by triangles.
- The x-axis represents %ACN (v/v) ranging from 0 to 100.
- The y-axis represents the range from 0 to 7000.
3.3.2 Effect of ACN on secondary structure of the BLG variants using far UV-CD

The secondary structural changes in BLG variants A and B at pH 2.0, 7.0 and 9.0 were monitored at various concentrations of ACN using far-UV circular dichroism (CD) spectroscopy. In the absence of ACN, the CD spectra of both BLG variants show the characteristic negative CD band at around 216 nm, typical of β-sheets proteins (Fig. 3.3.2 A-F, spectrum 1). These observations substantiate the earlier reports that β-sheets are predominant secondary structure in both BLG variants A and B in monomeric as well as dimeric forms (Dong et al., 1996).

At pH 2.0, no significant change in the far-UV CD spectra was observed until the ACN concentration reached 10 % (v/v) for BLG A (Fig. 3.3.2 A, spectrum 2) and BLG B (Fig. 3.3.2 B, spectrum 2). Above 10 % ACN, the CD spectra were transformed noticeably to typical α-helix spectra, with appearance of CD bands at 208 nm and 222 nm. At pH 7.0 and 9.0, the far-UV CD spectra of both BLG variants at 10% ACN are typical of β-sheet conformation as revealed by the single negative band at 213–218 nm (Fig. 3.3.2 C-F, spectrum 2). At concentrations of ACN >10%, the far-UV CD spectra of both variants show two negative CD bands at 208 nm and 222 nm, with more pronounced negative CD at 208 nm. Taken together, these results suggest that both BLG variants undergo nearly identical transitions from β-sheet to α-helix at all three pH values in presence of increasing concentration of ACN.

In order to further assess ACN-induced beta to alpha helical transitions, mean residue ellipticity at 222 nm of both BLG variants were monitored as a function of increasing concentrations of ACN. Figure 3.3.3 A, B and C show the mean residue ellipticity at 222 nm at pH 2.0, 7.0, 9.0, respectively. The β to α-helical transition appears to be more pronounced for BLG variants B at all pH values studied. The values of α-helical content of variant A in the presence of 40% ACN, as estimated from the molar ellipticity at 222 nm, are 43%, 32% and 28% at pH 2.0, 7.0 and 9.0, respectively. On the other hand, the values of α-helical content of variant B in the presence of 40% ACN are 56%, 36% and 36% at pH 2.0, 7.0 and 9.0, respectively. These observations suggested an increased amount of α-helical structure induction in BLG variant B compared to variant A in presence of 40% ACN at acidic, neutral and alkaline pH.
Effect of increasing concentration of ACN on secondary structure of BLG variants.

Far UV-CD measurements were made in the wavelength range (200-250 nm). Concentration of protein used was 10μM. Spectra 1-5 and G represent BLG in the presence of 0, 10, 20, 40, 60% ACN and 6M GnHCl, respectively. Far UV-CD spectra of BLG A at (A) pH 2.0, (B) 7.0 and (C) 9.0; and BLG B at (D) pH 2.0, (E) pH 7.0 and (F) pH 9.0, respectively are shown.

( —— ) BLG A
( —— ) BLG B
Fig. 3.3.3  Effect of increasing concentration of ACN on MRE of the BLG variants at 222 nm.

Plots of MRE vs. ACN concentration of BLG A and B at pH 2.0 (A), 7.0 (B) and 9.0 (C) are shown.

(—• —) BLG A  (■) BLG A in 6M GnHCl
(— ◀▼) BLG B  (□) BLG B in 6M GnHCl
3.3.3 Effect of ACN on conformation of the BLG variants

3.3.3.1 Intrinsic fluorescence

The conformational changes in BLG variants A and B at pH 2.0, 7.0 and 9.0 were monitored at various concentrations of ACN using intrinsic fluorescence measurements as a probe. In the absence of ACN (Fig. 3.3.4, spectrum 1), the intrinsic fluorescence spectra of both BLG variants at all the pH values studies showed characteristic λₘₐₓ at 332 nm. However, for both the variants, fluorescence intensity at pH 2.0 and 9.0 (Fig. 3.3.4 A, C, D and F, spectrum 1) was greater than that at pH 7.0 (Fig. 3.3.4B and E, spectrum 1). This behaviour can be explained on the basis of association of BLG into dimers and in turn leading to fluorescence quenching. At pH 2.0 and 9.0, both the variants are predominantly in monomeric form, while at pH 7.0 they are essentially dimeric (Sawyer and Kontopidis, 2000). Bovine BLG has two Trp residues, Trp 19 is situated in a hydrophobic environment at the bottom of the calyx formed by the antiparallel β-strands while Trp61 has been found to be adjacent to the strand involved in antiparallel interaction of the dimer (Divsalar et al., 2006). As suggested by Renard et al. (1998), the Trp61 is near the site of monomer-monomer association and may have contributed to the observed quenching of the fluorescence upon dimerization. However, λₘₐₓ seems to be insensitive to the association state of BLG indicating that the dimerization did not cause pH associated change in the conformation around either tryptophan side chains (Fig. 3.3.4 A-F, spectrum 1).

At pH 2.0 and 7.0, up to 20% (v/v) ACN no shift in λₘₐₓ was observed both for BLG A (Fig. 3.3.4 A and B, spectrum 3) and BLG B (Fig. 3.3.4 D and 4, spectrum 3). At 40% (v/v) ACN, a red shift occurred both for variant A (Fig. 3.3.4 A and B, spectrum 4) and variant B (Fig. 3.3.4 D and E, spectrum 4) which can be accounted for by the exposure of Trp and Tyr residues. But the red shift is less marked and FI higher than that of protein in presence of 6M GnHCl (Fig. 3.3.4, spectrum G). At pH 9.0, the red shift occurred in the range 10-40% (v/v) ACN for variant A (Fig. 3.3.4 C, spectra 2-4) and variant B (Fig. 3.3.4E, spectra 2-4). Beyond 40% (v/v) ACN, in all the cases, there was a gradual increase in FI with decrease in λₘₐₓ (spectra 5-6, Fig. 4).

Figure 3.3.5 and 3.3.6 show the effect of ACN concentration on fluorescence intensity and λₘₐₓ of the two variants when excited at 280 nm at pH 2.0 (Fig. 3.3.5 A, 3.3.6 A), 7.0 (Fig. 3.3.5 B, 3.3.6 B) and 9.0 (Fig. 3.3.5 C, 3.3.6 C), respectively. The FI vs. ACN profiles were almost comparable for the two variants at all the pH values (Fig.
Fig. 3.3.4 Effect of increasing concentration of ACN on intrinsic tryptophan fluorescence of the BLG variants.

BLG A at (A) pH 2.0, (B) 7.0 and (C) 9.0; BLG B at (D) pH 2.0, (E) pH 7.0 and (F) pH 9.0. Fluorescence measurements were made using $\lambda_{ec} = 280$ nm and $\lambda_{em} = 300-400$ nm. Concentration of protein used was 10 $\mu$M. Spectra 1-5 and G represent BLG in the presence of 0, 10, 20, 40, 60% ACN and 6M GnHCl, respectively. Intrinsic fluorescence spectra of BLG A at (A) pH 2.0, (B) 7.0 and (C) 9.0; and BLG B at (D) pH 2.0, (E) pH 7.0 and (F) pH 9.0, respectively are shown.

(—) BLG A

(—) BLG B
Fig. 3.3.5  **Effect of increasing concentration of ACN on fluorescence intensity of BLG variants at 332 nm.**

Plots of FI vs. ACN concentration of BLG A and B at (A) pH 2.0, (B) 7.0 and (C) 9.0 are shown.

( — • — ) BLG A  ( ■ ) BLG A in 6M GnHCl

( — ♦ — ) BLG B  ( ⊙ ) BLG B in 6M GnHCl
Fig. 3.3.6  Effect of increasing concentration of ACN on emission maxima (WLmax) of the BLG variants.

Plots of WLmax vs. ACN concentration of BLG A and B at (A) pH 2.0, (B) 7.0 and (C) 9.0 are shown.

(—○—) BLG A

(—●—) BLG B
3.3.5 A-C). The $\lambda_{\text{max}}$ however, first increased up to 30% (v/v) ACN and then decreased up to 70% (v/v) ACN (Fig. 3.3.6 A-C) for both the variants. This shift in $\lambda_{\text{max}}$ was more pronounced for variant A at pH 9.0 compared to variant B. The red shift at low ACN concentrations, which is less than that for GnHCl treated protein, suggests that although the protein variants have lost some of their tertiary structure, they are not completely denatured. A blue shift at higher ACN concentrations indicates decrease in polarity of Trp environment (Ahmad et al., 2010) and may be attributed to the creation of additional hydrophobic environment around Trp residues due to the formation of secondary structures, as discussed in far UV-CD section.

3.3.3.2 Near UV-CD

The tertiary structural changes in BLG variants A and B at pH 2.0, 7.0 and 9.0 were monitored at various concentrations of ACN using near UV-CD spectroscopy. At pH 2.0, no significant change in the near UV-CD spectra were observed until the ACN concentration reached 20% (v/v) for BLG A (Fig. 3.3.7 A, spectrum 3) and BLG B (Fig. 3.3.7 D, spectrum 3). Above 20% ACN the intensity of negative signals decreased (Fig. 3.3.7 A and D, spectra 4 and 5) approaching that of BLG in the presence of 6M GnHCl (spectrum G). At pH 7.0, the decrease in the negative CD signal was evident starting at 20% (v/v) ACN for BLG A (Fig. 3.3.7 B, spectrum 3) and beyond 20% (v/v) ACN for BLG B (Fig. 3.3.7 E, spectrum 4). At pH 9.0 and 10% (v/v) ACN the spectrum for BLG A (Fig. 3.3.7 C, spectrum 2) retained only some features of the spectrum of the native protein and at higher ACN concentration a decrease in the negative CD signals occurred until the spectral features approached that of BLG A in the presence of 6M GnHCl (Fig. 3.3.7 C, spectrum G). On the other hand, BLG B retained native like spectral features up to 10% (v/v) ACN (Fig. 3.3.7 F, spectrum 2) and a decrease in the CD signal began at 20% (v/v) ACN (3.3.7 F, spectrum 3).

A decrease in the negative CD signals reflects a less ordered tertiary structure. Figure 3.3.8 A-C show MRE at 293 vs. ACN concentration at pH 2.0, 7.0 and 9.0. It can be observed that at relatively low ACN concentration BLG A (Fig. 3.3.8 A) retained a compact structure at pH 2.0 while the variant B appeared to retain its structure at both pH 2.0 and 7.0 (Fig. 3.3.8 A and B). On the other hand at pH 9.0, both variants began to lose their tertiary structure at low ACN concentrations. The tertiary structure is completely lost at higher ACN concentration.
Fig. 3.3.7  Effect of increasing concentration of ACN on near UV-CD spectra of BLG variants A and B.

Near UV-CD measurements were made in the wavelength range 250-350 nm. Proteins' concentration used was 55.5μM. Spectra 1-5 and G represent BLG in the presence of 0, 10, 20, 40, 60% ACN and 6M GnHCl, respectively. Near UV-CD spectra of BLG A at (A) pH 2.0, (B) 7.0 and (C) 9.0; and BLG B at (D) pH 2.0, (E) pH 7.0 and (F) pH 9.0, respectively are shown.

( ) BLG A
( ) BLG B
Fig. 3.3.8 Effect of increasing concentration of ACN on MRE of the BLG variants at 293 nm.

Plots of MRE vs. ACN concentration of BLG A and B at (A) pH 2.0, (B) 7.0 and (C) 9.0, respectively are shown.

( —•— ) BLG A
( —◊— ) BLG B
3.3.3.3 ANS fluorescence

Figure 3.3.9 shows the ANS fluorescence profile of BLG variants at three pH values. ANS is known to bind to hydrophobic patches of proteins. In the absence of ACN (Fig. 3.3.9 A-F, spectrum 1) low FI was observed for both BLG variants thus suggesting the presence of very few exposed hydrophobic patches. In the presence of 6M GnHCl (Fig. 3.3.9 A-F, spectrum G), FI was further decreased as the denatured protein loses most of the ANS binding sites (Ahmad et al., 2005). At pH 2.0, 7.0 and 9.0, there was a very marginal increase in FI at 480 nm up to 20% (v/v) ACN both for variants A (Fig. 3.3.9 A-C, spectrum 3) and B (Fig. 3.3.9 D-F, spectrum 3). A more significant increase was however observed at 40% (v/v) ACN for BLG A (Fig. 3.3.9 A-C, spectrum 4) and BLG B (Fig. 3.3.9 D-F, spectrum 4) which gradually continued till 60% (v/v) ACN (spectrum 5). The higher affinity of ANS to the BLG variant in the presence of high ACN concentration suggests a remarkable increase in the number of solvent accessible non-polar clusters due to altered conformation and/or protein unfolding.

Figure 3.3.10 depicts the increase in FI at 480 nm with increase in ACN concentration. At pH 2.0, 7.0 and 9.0, BLG variant B showed greater FI than BLG A at identical ACN concentrations. Thus, it can be suggested that the exposure of hydrophobic patches resulting from the addition of ACN was more pronounced in the case of variant B, the difference being more marked at pH 7.0 and 9.0 than at pH 2.0.

3.3.4 Effect of acetonitrile on aggregation of the BLG variants

3.3.4.1 Transmission electron microscopy

As observed from Rayleigh scattering data, at pH 7.0 the aggregation of variant A (Fig. 3.3.1 A) and B (Fig. 3.3.1 B) commenced above 50% (v/v) concentration of ACN. The aggregated BLG samples incubated at 60%, 70% and 80% (v/v) ACN for 48 hrs were analysed under transmission electron microscopy. Figure 3.3.11 A-C shows the TEM images of BLG A samples with 60%, 70% and 80% (v/v) ACN. The TEM images of variant B at identical ACN concentrations are shown in figure 3.3.11 D-F. Annular protofibril-like structures were observed in samples at 60% (v/v) ACN (Fig. 3.3.11 A, D) and fibril-like aggregates were seen in samples at 70% (v/v) and 80% (v/v) ACN. In several pathological disorders and in various in vitro experiments, proteins have been known to turn into ‘amorphous aggregates’, without local order
Fig. 3.3.9 Effect of increasing concentration of ACN on ANS fluorescence of the BLG variants.

Fluorescence measurements were made using ($\lambda_{ex} = 380$ nm, $\lambda_{em} = 400$-500 nm). Concentration of protein used was 10μM. Spectra 1-5 and G represent BLG in the presence of 0, 10, 20, 40, 60% ACN and 6M GnHCl, respectively. ANS fluorescence spectra of BLG A at (A) pH 2.0, (B) 7.0 and (C) 9.0; and BLG B at (D) pH 2.0, (E) pH 7.0 and (F) pH 9.0, respectively are shown.

( ——— ) BLG A
( ——— ) BLG B
**Fig. 3.3.10** Effect of increasing concentration of ACN on fluorescence intensity of BLG variants at 480 nm.

Plots of FI vs. ACN concentration of BLG A and B at (A) pH 2.0, (B) 7.0 and (C) 9.0, respectively are shown.

- (♦) BLG A
- (■) BLG A in 6M GnHCl
- (◊) BLG B
- (□) BLG B in 6M GnHCl
Fig. 3.3.11  Microstructural features of fibrils obtained from the BLG variants by Transmission Electron Microscopy.

Images are of fibrils formed after 24 hrs of incubation at 37°C of BLG (10 μM) in 10 mM Tris HCl buffer pH 7.0 after exposing BLG variant A to (A) 60%, (B) 70% and (C) 80% ACN. Also, BLG B was exposed to (D) 60%, (E) 70% and (F) 80% ACN.
The native conformations of proteins have little tendency to aggregate because intermolecular interaction between the folded protein renders the majority of hydrophobic side chains, and the main chain amide and carbonyl groups, capable of forming strong hydrogen bonds, inaccessible for the formation of intermolecular interactions. Unfolding exposes such regions providing an opportunity for intermolecular interactions. In the case of BLG, the addition of moderate concentration of ACN was seen to enhance hydrophobic interactions and favour intermolecular hydrogen bonding resulting in protein precipitation.

### 3.3.5 Correlation of secondary structure formation with accumulation of hydrophobic patches

At pH 2.0, 7.0 and 9.0, with increasing ACN concentration an increase both in MRE at 222 nm (Fig. 3.3.3 A-C) and ANS binding (Fig. 3.3.10 A-C) was observed for BLG A as well as BLG B. ANS binding is enhanced when the protein forms compact conformation (Ahmad et al., 2010). Also, an increase in tryptophanyl FI at 332 nm (Fig. 3.3.5 A-C) and a blue shift (Fig. 3.3.6 A-C) of maximum wavelength was seen once the ACN concentration was increased beyond 30%. From these results, it can be suggested that the Trp residues are buried in more hydrophobic environment and that there has been a simultaneous formation of new secondary structure and hydrophobic clusters upon addition of ACN beyond 40% in case of both variants.

### 3.3.6 Correlation of helix induction with aggregation

On observing the spectral features of BLG A and B (Table 3.3.1, 3.3.2), obtained from far UV-CD data, helix induction could be clearly seen by monitoring the changes at 222 nm on addition of ACN. Data shown in tables 3.3.1 and 3.3.2 revealed considerable increase in helical content was found. Also, there occurred a concomitant loss of tertiary structure, as seen from near UV-CD data with increase in the ACN concentration between 0 and 80%. Organic co-solvents have been shown to promote aggregation in many proteins (Luo and He, 1999). From Rayleigh scattering data it was seen that at higher ACN concentration, aggregation of the protein occurred (Fig. 3.3.1 A and B). Aggregation of various other proteins in this solvent has also been reported earlier (Oliveira et al., 2009). The TEM images indicate the presence of annular protofibril-like structures (Fig. 3.3.11 A, B) and fibril-like structures (Fig. 3.3.11 C-F),
Table 3.3.1  Summary of different spectral properties of BLG A

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<tr>
<th></th>
<th>MRE at 222 nm&lt;sup&gt;a&lt;/sup&gt;</th>
<th>% helix&lt;sup&gt;b&lt;/sup&gt;</th>
<th>MRE at 293 nm&lt;sup&gt;c&lt;/sup&gt;</th>
<th>FI at 332 nm&lt;sup&gt;c&lt;/sup&gt;</th>
<th>λmax&lt;sup&gt;d&lt;/sup&gt;</th>
<th>FI at 480 nm&lt;sup&gt;d&lt;/sup&gt;</th>
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<tr>
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<tr>
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<td>-694</td>
<td>272</td>
<td>335</td>
<td>230</td>
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Table 3.3.2  Summary of different spectral properties of BLG B

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<tr>
<th></th>
<th>MRE at 222 nm&lt;sup&gt;a&lt;/sup&gt;</th>
<th>% helix&lt;sup&gt;b&lt;/sup&gt;</th>
<th>MRE at 293 nm&lt;sup&gt;c&lt;/sup&gt;</th>
<th>FI at 332 nm&lt;sup&gt;c&lt;/sup&gt;</th>
<th>λmax&lt;sup&gt;d&lt;/sup&gt;</th>
<th>FI at 480 nm&lt;sup&gt;d&lt;/sup&gt;</th>
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<td>-677</td>
<td>164</td>
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<tr>
<td>60% ACN</td>
<td>-19,851</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td><strong>pH 9.0</strong></td>
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<tr>
<td>Native</td>
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<td>23</td>
<td>-1,086</td>
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<td>-561</td>
<td>237</td>
<td>331</td>
<td>312</td>
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</tbody>
</table>

<sup>a</sup> MRE (deg.cm<sup>-1</sup>.dmol<sup>-1</sup>)

<sup>b</sup> Morrow et al., 2000

<sup>c</sup> Excitation wavelength 280 nm

<sup>d</sup> Excitation wavelength 380 nm
especially at higher concentrations of ACN. Apparently, BLG with predominantly helical structure begins to precipitate without the need for further conformational transition. The precipitation was more pronounced at pH 7.0 followed by that at pH 9.0 and lastly at pH 2.0 (Fig. 3.3.1 A and B).

The loss of tertiary structure suggested by near UV-CD data, exposure of Trp residues to the solvent as detected by intrinsic tryptophan fluorescence and increased ANS binding suggest that conformational changes affecting Trp19 environment in ACN treated protein involve the regions that also play a major role in terms of overall surface hydrophobicity of BLG and in BLG aggregation.

3.3.7 Effect of variation of pH on ACN induced conformational changes in BLG variants

From the spectral properties summarised in table 3.3.1 and 3.3.2 and respective figures, it can be observed that although the behaviour of the two variants in the presence of varying proportions of ACN was nearly identical, the magnitude of the changes induced by the solvent varied. While the induction of α-helical structure and exposure of hydrophobic patches was more pronounced for variant B, the loss of tertiary structure as indicated by MRE at 293 nm and shift in λmax upon excitation at 280 nm was more significant in case of the variant A than B. The two variants differ as already discussed, in two amino acid residues, Asp64 in A is replaced with Gly64 in B and Val118 in A has been found to be substituted with Ala118 (Sawyer and Kontopidis, 2000). It has also been suggested that the loss of the two methyl groups destabilize the core of the B variant relative to the A variant (Sawyer and Kontopidis, 2000). This might have implications on the ACN-induced unfolding patterns of the two variants.

The order of aggregation of the two variants in the presence of ACN was observed to be: pH 2.0 < pH 9.0 < pH 7.0. Also, at pH 2.0 helix induction occurred at relatively higher concentration of ACN than at pH 7.0 and 9.0. These observations suggest that the stability of BLG towards ACN induced unfolding is greater at acidic pH than at either neutral or alkaline pH.

There exists evidence that ACN which is a water miscible solvent with amphiphilic nature, is capable of stripping water molecules away from the protein surface (Cardoso et al., 2009). Thus, it can be assumed that the primary action of ACN
on BLG could have been perturbation of hydration structure of the protein, and simultaneous loss of non-covalent interactions of BLG molecules with water resulting in disruption or weakening of its natively ordered state (Cardoso et al., 2009), finally leading to protein precipitation at much high ACN concentrations. While stripping away water, it is also conceivable that the polar and relatively small sized ACN molecules penetrate into protein surface crevices. Also, the solvent is known to decrease the dielectric constant of the medium surrounding the protein thereby facilitating formation of additional hydrogen bonds among the polar amino acids located on protein surface (Safarian et al., 2006). This property of the solvent may explain the transformation of secondary structure elements of the BLG variants. Interestingly, in the presence of ACN, BLG aggregation increases simultaneously with increase in α-helicity. Proteins with various types of structures; like α-helical: apolipoprotein A1 (Rousseau et al., 2006), PrPc (Merlini and Bellotti, 2005), β-sheet: crystallins (Bratosiewiez-Wasik et al., 2004), transthyretin (Rousseau et al., 2006), α+β: lysozyme and gelsolin (Hirschfeild, 2004), α/β: cystic fibrosis trans-membrane regulator (Sandilands et al., 2002) or natively unfolded: Aβ peptide and tau (Welch, 2004) etc., have been reported to exhibit aggregation (Jacobson et al., 2005) in presence of ACN. Although β-structures are mostly associated with the formation of aggregation in protein, but involvement of α-helical aggregates have also been reported in case of several proteins (Barghorn et al., 2004). Kunjithapatham et al. (2005) reported that TFE favours the assembly of tau into α-helical aggregates and the aggregation process involves the arrangement of preformed α-helices into coiled-coils.

It has earlier been reported that bovine BLG has a high propensity for α-helical conformation (Nishikawa and Noguchi, 1991; Shiraki et al., 1995; Hamada et al., 1995; Kuroda et al., 1996). Several authors have shown that when non-local interactions between amino acid residues distant from each other in the sequence are removed, the helical structure of the protein attains stability (Shiraki et al., 1995; Hamada et al., 1995; Kuroda et al., 1996; Hamada and Goto, 1997; Kuwata et al., 1999). Other refolding studies have also shown that the non-native α-helical structure play important role in forming the protein’s native β-sheet structure (Hamada and Dobson, 2002). In the present work, analysis of the conformational properties of the bovine BLG variants A and B, accumulated during equilibrium unfolding induced by ACN at three different pH values was undertaken. The two variants showed similar behaviour qualitatively,
under the experimental conditions chosen, though the magnitude of the changes induced varied to some extent. The transition from native β-sheet to α-helical form was analysed by far UV-CD spectral measurements made at varying ACN concentrations. ACN is known to induce β-sheet in some proteins (Srisailam et al., 2002) due to its higher polarity than alcohols in water, which provides a favourable environment for the intermolecular beta sheets (Smythe et al., 1995). But in the case of BLG which is predominantly β-sheeted, non-native helix was found to be the predominant structure at higher ACN concentration. ANS binding data confirmed that surface hydrophobicity increased upon increasing ACN concentration. RLS data together with ANS binding suggest that increased hydrophobic interactions contribute significantly to the formation of the BLG aggregates. Aggregation appears to be pH dependent, following the order 7.0 > 9.0 > 2.0, further providing evidence of the exceptional stability at pH 2.0 (Sawyer and Kontopidis, 2000; Madureira et al., 2007). Near UV-CD spectra of BLG exposed to varying concentration of ACN indicate that the solvent induces gradual structural alterations at low concentration while drastic changes occur at higher concentration. Intrinsic fluorescence data showed significant influence of ACN on the environment surrounding Trp19. Trp 19 residue, present at the bottom of the protein calyx, is known to be the major contributor to the spectroscopic properties of the protein (Renard et al., 1998).

It is now well documented that several pathological conditions involve accumulation of aggregated protein forms (Bellotti et al., 2000; Fandrich et al., 2001; Bucciantini et al., 2002). Numerous proteins tend to attain comparable aggregated structures in vitro under appropriate conditions either by loss or transformation of secondary structure elements (Rasmussen et al., 2007). Several reports exist which contain evidence of transformation of BLG into fibrillar structures in vitro (Hamada and Dobson, 2002) mediated by chemical or physical denaturation of the protein (Kuwata et al., 2001; Lametti et al., 2002). Studies on BLG have generated interesting data and is considered a model to study mechanism of α-helix to β-sheet transition of proteins, which may have implications in understanding the pathology of diseases like Alzheimer’s (Kuwata et al., 1999). In the present study we have monitored the effect of ACN, a routinely used solvent in RP-HPLC, on unfolding of BLG variants A and B at three different pH 2.0, 7.0 and 9.0 with the aim of gaining insight into the nature of protein folding pathway, stability of the protein in acidic, neutral and alkaline
conditions and aggregation behaviour of the protein in the presence of this organic solvent.