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Supplementary Information

New insight into the alcohol induced conformational change and aggregation of alkaline unfolded state of bovine β-lactoglobulin

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2.1. Materials Bovine β-lactoglobulin (β-lg) was isolated and purified from cow milk as described earlier. [33] Methanol and different fluorescent probes, viz., 8-anilinonaphthalene, 1-sulfonic acid ammonium salt (ANS), Congo Red (CR) as well as Thioflavin T (ThT) were obtained from Sigma Chemical Co. (St. Louis, USA) and used as received without further purification. 2-Propanol (IP), *t*-butanol, sodium phosphate dibasic, glycine and KOH were purchased from Merck (Mumbai, India) (all were AR grade). 2, 2, 2-trifluroethanol (TFE) (spectroscopic grade) was purchased from Spectrochem Pvt. Ltd. (India).The other chemicals used were of highest purity available.

2.2. Methods and instrumentation

2.2.1. Protein samples preparation

All the measurements were carried out at room temperature. Since the extinction coefficient of β-lg ($0.96 mg^{-1}ml^{-1}cm^{-1}$ at 280 nm) is known, different concentrations of protein samples were prepared by dissolving β-lg samples in buffer solution of pH 10.5. All the experiments for native β-lg were performed in 10 mM sodium phosphate buffer pH 7.0. For the study at alkaline pH, β-lg was dissolved in 3 mM glycine-KOH buffer of pH 10.5 respectively to give a stock solution of (271 μM) in each case. To the stock protein solutions, different volumes of buffer were added first, followed by the addition of methanol, 2-propanol, t-butanol and TFE (assumed to be 100%, v/v) to get a desired concentration of co-solvent in each case. The final solution mixture (1.0 ml) was incubated for 4 h at room temperature before each experiment. The required protein concentrations were adjusted in each experiment.

2.2.2. Intrinsic fluorescence study

Fluorescence measurements were performed on a Shimadzu spectrofluorimeter (Shimadzu 5301 PC). The fluorescence spectra were collected at 25 °C using 1 cm path-length cell and protein concentration 13.6 μM in absence and presence of different non-fluorinated and fluorinated alcohols. The excitation and emission slits were set at 5 nm. Intrinsic fluorescence spectra were recorded in the wavelength region 310 to 400 nm after exciting the protein sample at wavelength of 295 nm.

2.2.3. 1-Anilinonapthalene-8-sulfonate (ANS) fluorescence studies to monitor the hydrophobicity

Exposure of hydrophobic patches in protein during the aggregation process was monitored using polarity sensitive fluorescent probe 1-Anilinonapthalene-8-sulfonate (ANS). [34]A stock solution of ANS was added to each aliquot of β-lg solution (both in absence and presence of different non-fluorinated and fluorinated alcohols) so that the final ANS concentration in each aliquot was 30 μM. Typically, ANS concentration was 50 molar excess of protein concentration. The ANS-fluorescence intensities were measured using Shimadzu RF-5301 PC with excitation at 370 nm [35, 36] and scanning the emission wavelength from 400 nm to 650 nm. Slit widths were set at 5 nm for both excitation and emission. Each spectrum was blank corrected. Data points were the average of triplicate measurements.

2.2.4. Thioflavin T (Th T) assay

ThT is a dye which shows enhanced fluorescence at 480 nm when bound to amyloid fibrils. [37] Thus to investigate and compare the aggregates formed by β-lg in presence of various non-fluorinated and fluorinated alcohols, the following assay was employed. Briefly 250µL of β-lg samples having concentration 1mg/ ml was taken. It was then added to 40 µL Th T solution (stock 3.13 mM ThT in 10 mM sodium phosphate buffer, pH 7.0) containing a mixture of buffer and varying amount of different alcohols, mixed thoroughly and incubated for 30 min. [38] The final concentration of protein was 6.8 µM while the concentration of Th T was 30 µM. The assay solution was excited at 450 nm [39] and the emissions were measured over the range 460 to 600 nm. Slit widths for both excitation and emission were kept at 5 nm. Three replicates were performed and the data were averaged.

2.2.5. Congo red assay

The formation of aggregates in presence of alcohols was probed by measuring the shift in absorbance of Congo red in the region 400–650 nm. For this experiment, 240µl (27.2µM) aliquots of the protein solutions were withdrawn and mixed with 260µl of a solution containing 40µM Congo red solution. Final volume (2 ml) was adjusted with 3mM glycine-KOH buffer of pH 10.5 and varying concentrations of various non-fluorinated and fluorinated alcohols. [40]

2.2.6. Analysis of secondary structures by CD Spectroscopy

To investigate the conformational changes of β-lg in presence of different non-fluorinated and fluorinated alcohols, circular dichroism measurements were carried out on a Jasco Spectropolarimeter (J-815) at 20°C in the far-UV region (190-260nm) using the rectangular cell of 1mm path length. The β-lg solutions equilibrated separately with non-fluorinated and fluorinated alcohols having protein concentration 13.6 µM. All the spectra are average of three scans. The final spectrum was obtained after the subtraction of corresponding solvent spectrum. The far UV-CD curves were fitted into a curve-fitting program CDNN 2.1 to determine the amount of secondary structures present in β-lg in presence of methanol, 2-propanol, *t*-butanol and TFE acting as co-solvents.

2.2.7. Dynamic Light Scattering (DLS) measurements

The diffusion of nano sized particulates in solution induces fluctuations in the intensity of the scattered light. DLS detects these fluctuations using an auto correlator on a microsecond time scale and is used to analyze the distribution of the molecules and supramolecular aggregates as it is very sensitive to particle size. [41] Different sizes of molecules in the solution can be observed in different peaks provided their sizes vary sufficiently. In our experiment, DLS measurements were performed with β-lg solutions in absence and presence of Methanol, 2-propanol, *t*-butanol and TFE employing Zetasizer Nanos (Malvern Instrument, U.K.) equipped with 633 nm laser and using 2 ml rectangular cuvette (path length 10 mm). Measurements were done at 20°C taking 250 µl of β-lg sample in 1.75 ml 3mM glycine-KOH buffers of pH 10.5.Then different amounts of non-fluorinated and fluorinated alcohols were mixed thoroughly and allowing to equilibrate the solutions. The time-dependent auto correlation function was acquired with twelve acquisitions for each run. Each data is an average of five such acquisitions.

2.2.8 Monitoring of secondary structure of β-lg during aggregation by FT-IR spectroscopy

For FT-IR measurements, 50 µl β-lg sample solutions (in absence and presence of Methanol, 2-propanol, *t*-butanol and TFE) having concentrations (1087 µM) were taken in a microcon filter device and diluted with 200 µl of D_2O. It was then quickly centrifuged at 4000×g for 8 min until the volume reached ~ 50 µl. After that 200 µl of D_2O was added again and centrifuged for another 8–10 min. This process of D_2O exchange was repeated 3-4 times. [39] Finally, the D_2O exchanged β-lg samples were placed between two CaF_2 windows separated by a 50 µm thick teflon spacer. FT-IR scans were collected in the range of 1550–1750 cm^{-1}at a resolution of 2 cm^{-1}in N_2 environment using a Spectrum 100 FT-IR spectrometer (Perkin Elmer). Spectrum of D_2O at pD 7.0 was collected and subtracted from sample spectrum.

2.2.9 Morphological studies with SEM and TEM

Field emission scanning electron microscope (FESEM)

The morphology of the aggregates of β-lg generating from U_A state in various alcoholic co-solvents at different concentrations were investigated using FE-SEM (Hitachi S-4800, JAPAN) operating with a voltage of 20kV. For this study the protein sample (U_A state) was incubated with MeOH, *i*-PrOH, *t*-BuOH and TFE and the concentrations were maintained at 10 µM. One drop of the

sample solution was taken on a glass slide. It was dried by slow evaporation in open air and then under vacuum and gold coated for imaging.

Transmission electron microscopy (TEM)

The morphology and size of the aggregates of β-lg obtained from U_A state after incubation separately with non-fluorinated and fluorinated alcohols were investigated by high resolution transmission electron microscopy (Jeol-HRTEM-2011, Tokyo, Japan) with an accelerating voltage of 80–85 kV in different magnifications. The sample solutions were diluted 50 times in 3mM glycine-KOH buffer of pH 10.5. A droplet of the diluted sample was put on a carbon coated copper grid of mesh size 300C (Pro Sci Tech). After 20s the droplet was removed with a filter paper followed by a droplet of 2% uranyl acetate (Sigma, Steinheim, Germany) solution put on the grid and finally removed after 15 s and left for air dry and used for imaging purpose. Before taking the image all the samples were incubated 6 h.

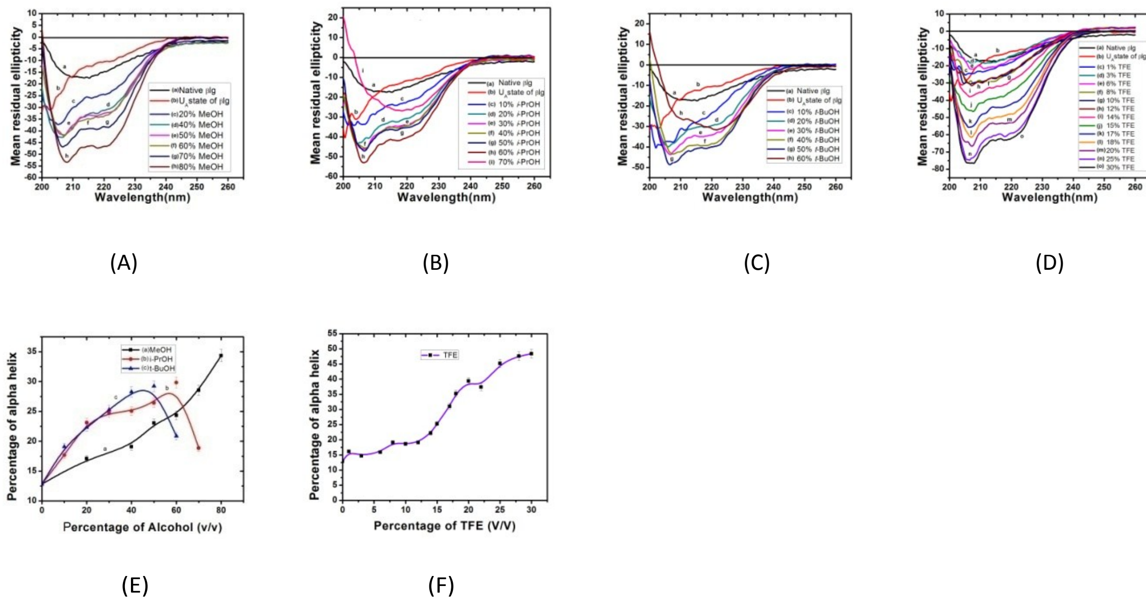


Fig.1. Far-UV CD spectra of β-lg (13.6 μM) in native state in 10 mM sodium phosphate buffer pH-7.0 (curve a) and in alkaline unfolded state (curve b) in presence of 20%, 40%, 50%, 60%, 70% and 80% (v/v) MeOH (curve c-h) (A), 10-70% (v/v) *i*-PrOH (curve c-i) (B), 10-60% (v/v) *t*-BuOH (curve c-h) (C) and with 1%, 3%, 6%, 8%, 10%, 12%,14%,15%,17%, 18%, 20%,25% and 30% (v/v) TFE (curve c-o) (D) at pH 10.5 in 3mM glycine-KOH buffer. Percentage of α-helix formed at 222 nm in the presence of MeOH, *i*-PrOH and *t*-BuOH (E) and TFE (F) in alkaline unfolded β-lg. The far-UV CD spectra were recorded between 200 nm and 260 nm and the path length was 1 mm.

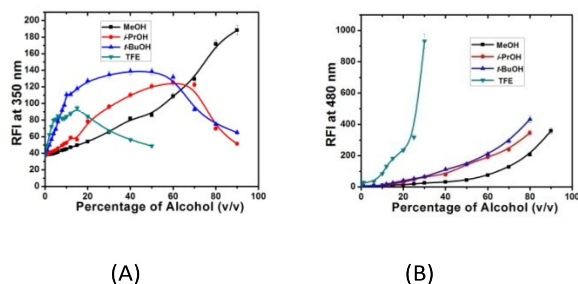
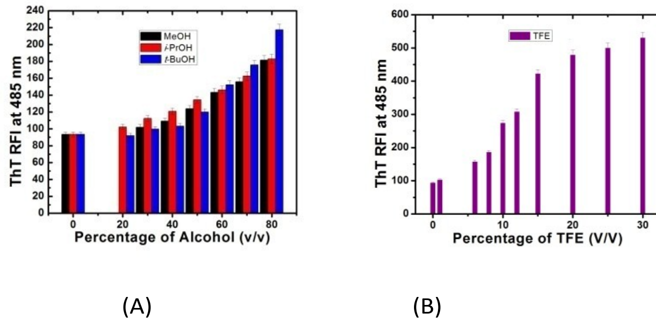


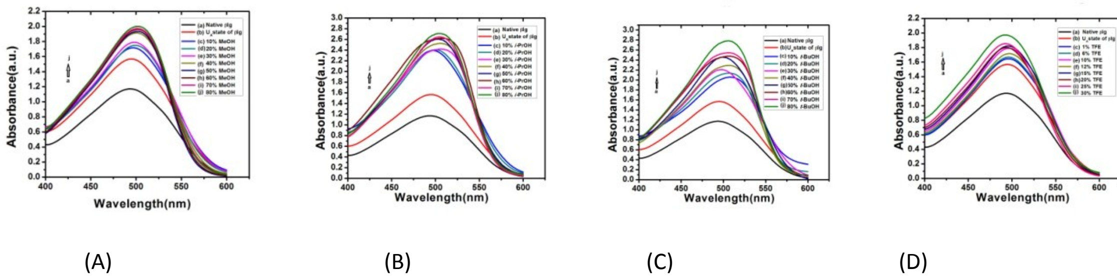
Fig. 2.(A) Normalized relative fluorescence intensity of tryptophenyl residues (emitted at 350 nm) of alkaline unfolded β-lg (pH 10.5) with increasing concentrations of MeOH (a), *i*-PrOH (b), *t*-BuOH (c) and TFE (d), when the sample was excited at 295 nm. Protein concentration was 13.6 μM. (B) Relative fluorescence intensity at 480nm of alkaline (pH

10.5) unfolded β-lg-ANS complex with increasing concentration of methanol (a), isopropanol (b), *t*-butanol (c) and TFE (d) after exciting at 370 nm.



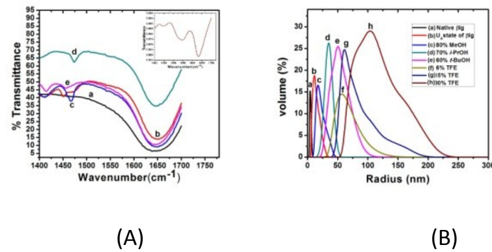
(A) (B)

Fig.3. Bar diagram of the end-point ThT intensity versus (A) MeOH, *i*-PrOH and *t*-BuOH concentrations (B) TFE concentrations in Th T assay to study the aggregation of β-lg in 3mM glycine-KOH at pH 10.5 buffer. Fluorescence emissions were monitored in the wavelength range 460–600 nm after excitation at 450 nm. Standard deviations are within the range of ±3.0. A control experiment of ThT without β-lg has been performed.



(A) (B) (C) (D)

Fig.4. Congo red absorption spectra of β-lg in native state in 10 mM sodium phosphate buffer pH-7.0 (curve a) and in alkaline unfolded state (curve b) in 3mM glycine-KOH buffer at pH 10.5 in presence of (10-80%) (v/v) of MeOH (curve c-j) (A),*i*-PrOH (curve c-j) (B), *t*-BuOH (curve c-j) (C) with interval of 10 and in presence of 1%, 6%, 10%, 12%, 15%, 20%, 25% and 30% TFE (curve c-j) (D). The absorption spectra were recorded from 400 to 600 nm. The protein concentration was 27.2 µM.



(A) (B)

Fig.5. (A) Fourier transform infra red (FTIR) spectra of alkaline unfolded β-lg (curve a) and native β-lg (inset) , in the presence of 80% (v/v) MeOH (curve b), 70% (v/v) *i*-PrOH (curve c), 60% (v/v) *t*-BuOH (curve d) and 30% (v/v) TFE (curve e) respectively at pH 10.5 in 3mM glycine-KOH. Protein concentrations were 1087 µM. Each spectrum is an average of 32 scans in D_2O solvent at 25^0C. (B) Number-particle size distribution profile of native β-lg (curve a), alkaline unfolded β-lg (curve b) in the presence of 80% (v/v) MeOH (curve c), 70% (v/v) *i*-PrOH (curve d), 60% (v/v) *t*-BuOH (curve e) and 6% (v/v), 15%(v/v) and 30% (v/v) (curve f-h) TFE respectively in same buffer. Each of these spectra is an average of 48 scans.

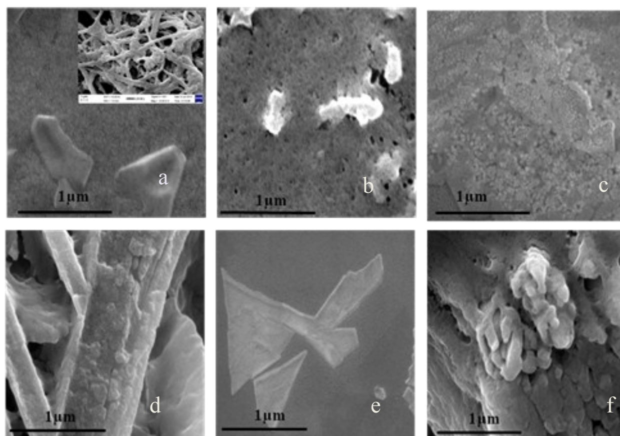


Fig.6. FE-SEM images showing the formation of distinct self assembled structure of β-lg at pH 10.5 and at : β-lg in absence of any alcohol(a) and native β-lg(inset), worm-like aggregates of 50-60 nm diameter in 80% (v/v) MeOH (b), smaller spherical nanoparticles in 70% (v/v) *i*-PrOH (c), long sheet-like aggregates in 60% (v/v) *t*-BuOH (d), flake-like and nanotube-like morphology with 15% (v/v) (e) and 30% (v/v) TFE (f).

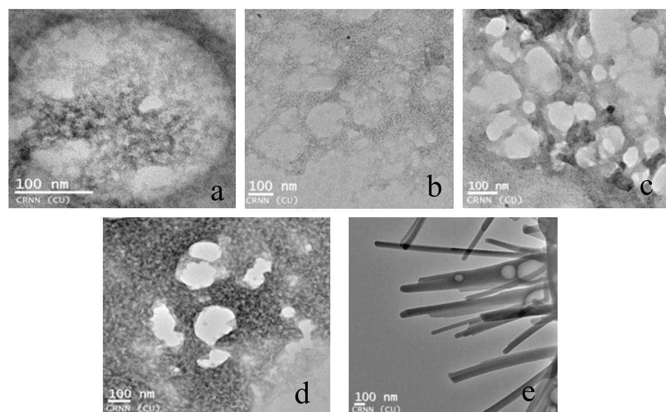


Fig.7. Selected TEM images of β-lg aggregates at pH 10.5 and at 30^{0}C: β-lg in absence of any alcohol (a), amyloid fibrillar network in 80% (v/v) MeOH (b), nanovesicular-like structures in 70% (v/v) *i*-PrOH (c), spherical shaped nanoparticles in 60% (v/v) *t*-BuOH (d), formation of nanotube-like structures of the average thickness of 5 nm in 30% (v/v) TFE (e). Images were taken after 6 h incubation and the protein concentrations were 10μM.