RESULTS AND DISCUSSION
Chapter 3

HOLDING CHAPERONE ACTIVITY OF E. COLI SMALL HEAT-SHOCK PROTEINS IbpA AND IbpB: AN BIOPHYSICAL APPROACH
In vitro holdase activity of *E. coli* small heat-shock proteins IbpA, IbpB and IbpAB

The proteins IbpA and IbpB were so named because they were first identified as components of inclusion bodies, formed due to aggregation of over expressed recombinant proteins in *E. coli* cells [Allen et al, 1992]. They were later found to be present in *E. coli* cells under heat shock conditions [Laskowska et al, 1996]. Like other *E. coli* heat shock genes, ibpA and ibpB genes were also transcribed by the sigma-32-containing RNA polymerase [Chuang et al, 1993]. Over expression of IbpA and/or IbpB enhanced thermo tolerance of *E. coli* cells [Kitagawa et al, 2000], whereas deletion of the genes caused a two-fold increase of aggregated proteins and a ten-fold decrease of cell viability upon long term exposure at 50°C [Kuczynska-Wisnik et al, 2000]. IbpA and IbpB showed 48% homology of amino acid sequence and belonged to the family of small heat shock proteins (sHsps) widely distributed among prokaryotes and eukaryotes. The sHsps were characterized by their low monomeric molecular mass (12 – 43 kDa), oligomeric structures and the presence of a conserved stretch of approximately 100 amino acid residues C-terminal “α-crystallin” domain [Haslbeck et al, 2005; Narberhaus et al, 2002]. IbpA and IbpB of monomeric molecular mass 15.7 and 16.3 kDa respectively, dissociated reversibly upon prolonged incubation at 50°C from multimers of 2-3 MDa to monomers in the case of IbpA and to oligomers of 650–700 kDa in the case of IbpB [Kitagawa et al, 2002; Shearstone et al, 1999]. Several in vitro experiments on different enzymes revealed that IbpA and IbpB functioned as ATP-independent molecular chaperones and protected unfolded, denatured proteins from irreversible aggregation [Shearstone et al, 1999; Veinger et al, 1998; Ratajczak et al, 2009]. Substrates bound to IbpB preserved their “folding-competent state” and were delivered for subsequent refolding to the ATP-dependent DnaKJE-ClpB bi-chaperone system [Mogk et al, 2003a; 2003b]. There were also reports that the presence of IbpA alone during substrate denaturation did not increase substrate reactivation by the DnaKJE-ClpB bi-chaperone system, rather substantial inhibition of the process was observed; whereas IbpB associated with
aggregates at high temperature due to its interaction with IbpA and released the IbpA-mediated inhibitory effect [Ratajczak et al, 2009, Matuszewska et al, 2005].

The background of the above facts tempted us to investigate – which one of the IbpA and IbpB was more active as holdase and how the activity of one was modulated by the presence of the other. Our results, on the inhibition of deactivation and aggregation of the heat-denatured protein lactate dehydrogenase in the presence of IbpA, IbpB and (IbpA+IbpB), showed that the activity of IbpA was less than that of IbpB and the activity of IbpA and IbpB in combination was more than the activity of each of them. This study further demonstrated the use of uncommon modern instruments like micro-viscometer, particle size analyzer (working principle being dynamic light scattering) and atomic force microscope in the protein folding study, about which there are fewer reports [Gast et al, 1992; Hagen et al, 2010; Best et al, 2003].

3.1 MATERIALS & METHODS

3.1.1 MEDIA, REAGENTS AND CHEMICALS

The nutrient broth for bacterial cell growth was purchased from Hi-media, India; the rabbit muscle L-lactate dehydrogenase (LDH), Tris-HCl, NADH, sodium pyruvate, and other fine chemicals (viz., antibiotic Kanamycin, IPTG, PMSF etc.) from Sisco Research Laboratories, India; Ni2+-NTA agarose from QIAGEN, Germany.

3.1.2 ISOLATION AND PURIFICATION OF IbpA AND IbpB FROM E. coli

The proteins IbpA / IbpB were isolated from E. coli strain BL21(DE3), containing his-ibpA / his-ibpB clones into pET28+ vector, and subsequently purified as described in [Matuszewska et al, 2005; Sambrook 2001].
ISOLATION AND PURIFICATION OF IbpA AND IbpB

Purified IbpA & IbpB proteins were isolated from *E. coli* strain BL21(DE3) [Matuszewska et al, 2005], containing his-ibpA / his-ibpB clones into pET28+ vector. Cells of strain BL21(DE3) were initially grown at 37°C in 1 L nutrient broth, supplemented with 50 µg/ml Kanamycin, up to bacterial (OD)600nm = 0.6-0.8. Isopropyl-β-D-thio-galactopyranoside (IPTG) was then added to the grown culture at a final concentration of 1 mM and the cells were allowed to grow further at 37°C for an additional 2 hr for induction of IbpA / IbpB. Cells were then harvested by centrifugation at 7000 × g for 15 min, washed with 10 ml of buffer A (50 mM Tris-Hcl, pH 7.4 and 50 mM NaCl), finally suspended in 20 ml of sonication buffer (buffer A + 1 mM phenylmethylsulfonyl fluoride [PMSF]) and sonicated using Cole-Parmer 4701-series ultrasonicator (micro-tip, level 6, 50% duty cycle, 12 pulses each of 1 min at intervals of 1 min) to disrupt the cells. Cellular debris was removed by centrifugation at 12000 x g for 45 min. The supernatant was loaded onto a 3 ml Ni2+-NTA-agarose column, pre-equilibrated with buffer A containing 15 mM imidazole. The column was subsequently washed with 20 ml of buffer A + 15 mM imidazole at a rate 0.4 ml/min at 4°C. Nickel-bound His6-tagged IbpA / IbpB protein was finally eluted with 30 ml of 15-150 mM imidazole gradient in buffer A. The eluted protein was dialyzed against two changes of 1 L of buffer A at 4°C. The dialyzed protein was then concentrated to 1 mg/ml using Centriplus-100 concentrator (Amicon) of molecular mass cut-off 10 kDa (this operation led to the removal of contaminating low molecular mass proteins), and the resulting protein was ~ 95% pure.

Previous studies reported that about 60% of the overproduced IbpA was found in the fast-sedimenting fraction of cellular extracts containing membranes and aggregated proteins, and the remaining 40% was in the soluble fraction. Therefore, in case of IbpA, after centrifugation of the sonicated extract, both the supernatant and the pellet were collected; the supernatant was individually loaded on to Ni2+-NTA column and the pellet was dissolved in 6 M urea and stirred overnight at 25°C to release the bound IbpA from the aggregates. The stirred sample was centrifuged and the supernatant was dialyzed...
against two changes of 1 L of buffer A at 4°C to remove out urea. The dialyzed sample was then loaded onto Ni2+-NTA column and the subsequent steps were followed to obtain purified and concentrated IbpA protein, as discussed in case of IbpB.

To check the purity of IbpA/IbpB, they were run through 12% SDS-polyacrylamide gel, as described in Appendix A-1 [Chattopadhyay et al, 1994]. For this, 20μl of purified IbpA/IbpB was mixed with 4 μl of 6X sample loading buffer and finally 16 μl of the mixture was loaded on the gel. After the electrophoresis was over, the gel was stained with Coomassie Brilliant Blue to view the protein bands and concentration of the protein was determined by using Bradford method described in Appendix A-2.

3.1.3 ASSAY OF HOLDING CHAPERONE PROPERTY OF IbpA AND IbpB

3.1.3.1 BY THE TECHNIQUE OF SPECTROPHOTOMETRY:

The ‘holdase’ or the holding chaperone activity of IbpA or IbpB was studied by measuring spectrophotometrically the extent of inhibition of (a) inactivation and (b) aggregation of heat-denatured tetrameric L-LDH, allowing the denaturation in the presence of IbpA or IbpB. The dehydrogenase activity of native LDH was assayed by measuring the rate of conversion from NADH to NAD+ [Badcoe et al, 1991; Chattopadhyay et al, 1994] at 30°C [appendix A-3]. The (absorbance)340nm of the assay mixture (100 mM Tris/HCl of pH 7.5, 5 mM sodium pyruvate, and 250 μM NADH) was measured at different intervals of time in a temperature-controlled spectrophotometer (Shimdzu, UV1800). The decrease in (absorbance)340nm with time signified enzyme activity through the disappearance of NADH, because NADH but not NAD+ had absorption maxima at 340 nm. On the other hand, the heat-denatured aggregation of LDH was monitored by measuring the (absorbance)360nm of LDH solution at different intervals of time at 60°C [Matuszewska et al, 2005]. The increase in (absorbance)360nm with time signified protein aggregation through the appearance of turbidity in the original transparent protein solution. For activity study, the working concentration of LDH was 0.1 mg/ml and that of IbpA or IbpB was 0.5 mg/ml. For aggregation study, the concentrations were 0.2 mg/ml and 1.0 mg/ml respectively, all dissolved in 50 mM tris buffer of pH 7.5. To investigate the effect of the
joint action of IbpA and IbpB, each was added separately at a concentration of 0.5 mg/ml in case of activity study and at a concentration of 1.0 mg/ml in case of aggregation study.

3.1.3.2 BY THE TECHNIQUE OF SPECTROFLUORIMETRY:

The change in the intrinsic fluorescence [Schmid 1997] of LDH, with its denaturation by heat in the absence and presence of IbpA, IbpB or (IbpA+IbpB), was measured in a Varian (Cary eclipse) fluorescence spectrophotometer. The sample was excited at 280 nm and the fluorescence emission spectra were recorded from 300 to 500 nm, keeping both slits at 7.0 nm. In order to eliminate the contribution of IbpA/IbpB/(IbpA+IbpB), baseline correction (i.e. by adjusting the fluorescence level of each of IbpA/B/(A+B) to zero) was done and then the measurement on LDH was performed.

3.1.3.3 BY THE TECHNIQUE OF MICRO-VISCOMETRY:

The measurement was done using Brookfield (DV II + Pro v-6.3) micro-viscometer at LV external mode. The viscometer employed the principle of rotational viscosity, according to which the amount of viscous drag in a fluid was proportional to the amount of torque required to rotate a spindle through the fluid and thus measured the viscosity of the fluid. The change in the intrinsic viscosity of L-LDH, with its denaturation by heat in the absence and presence of IbpA, IbpB or (IbpA+IbpB), was measured.

3.1.3.4 BY THE TECHNIQUE OF DYNAMIC LIGHT SCATTERING:

The change in the hydrodynamic size of LDH molecules, under the condition of denaturation by heat in the absence and presence of IbpA or IbpB or both, was determined using a particle size analyzer (Malvern, Nano ZS). Sample was illuminated here with 633nm laser and the time-dependent fluctuations in the intensity of scattered light from suspended particles undergoing random, Brownian motion were measured by an avalanche photodiode, which determined the diffusion coefficient and finally provided the size distribution of the particles by using Stoke-Einstein equation [Zetasizer nano user manual].
3.1.3.5 BY THE TECHNIQUE OF ATOMIC FORCE MICROSCOPY:

Veeco dl-inova atomic force microscope (AFM) having large area scanner was used for the visualization of L-LDH conformation at different temperatures like 30°, 52° and 60°C in the absence and presence of lbpA/lbpB/(lbpA+lbpB). For this, protein sample at the working concentration in tris buffer of pH 7.5 was allowed to fix on mica surface for 12 hr at vacuum desicator, followed by a washing step by the same tris buffer containing low concentration of imidazole (25mM), which has strong affinity for histidine and thus removed his-lbpA and his-lbpB from the samples. The samples were then dried well before imaging at tapping mode by a phosphorus (n) doped silicon probe of spring constant 20-80 N/m.

3.1.4 MOLECULAR DYNAMICS SIMULATION OF RABBIT MUSCLE L-LDH AT 52°C

3.1.4.1 PDB FILE PREPARATION:

The rabbit muscle L-lactate dehydrogenase (PDB ID: 3H3F) was downloaded from PDB (http://www.rcsb.org/pdb) [Rose et al, 2011] and was found to contain two tetramers in its crystal structure. From the downloaded structure, one of the two tetramers, hetero-atoms and water molecules associated with the crystal were removed. Conformers were deleted on the basis of occupancies and further the occupancies were corrected. The missing atoms and nomenclature issues were fixed with Accelrys DS Studio and Swiss-PdbViewer [Guex et al, 1997].

3.1.4.2 MOLECULAR DYNAMICS (MD) SIMULATION:

MD simulation was performed using GROMACS 4.5.4 (Groningen Machine for Chemical simulations) [Hess et al, 2008] simulation packages employing OPLS-aa (Optimized Potentials for Liquid Simulations-all atom) force field [Jorgensen et al, 1988]. The protein was protonated at pH 7.5 and solvated by explicit SPC/E (extended simple point charge)
water model [Berendsen et al, 1987] in cubic boxes maintaining a minimum 10 Å distance from the cube edges. Counter ions (Cl- and Na+) were added by replacing water molecules to achieve a neutral simulation cell. The whole system was then minimized using a steepest descents integrator [Van der Spoel et al, 2011] either until the maximum force was less than 1000 kJ mol-1nm-1 on any atom or until additional steps resulted in a potential energy change of less than 1 kJ mol-1.

A 100 ps NVT [constant number of particles (N), system volume (V) and temperature (T)] equilibration was performed at 300 K with position restraints applied to all the backbone atoms in order to relieve any bad contacts at the side chain-solvent interface. The velocity rescale thermostat [Bussi et al, 2007] was used with a temperature coupling time constant (τT) of 0.1 ps. All bond lengths were constrained using the linear constraint solver (LINCS) algorithm, [Hess et al, 1997] which allowed for a 2 fs time step. Long-range electrostatic interactions were approximated using the Particle Mesh Ewald (PME) method [Darden et al, 1993] with a fourth-order spline interpolation and a 0.15 nm Fourier grid spacing. The short range non-bonded interactions were defined as van der Waals and electrostatic interactions between particles within 10 Å.

After the primary equilibration, the position restraints were lifted and the velocity rescale thermostat was raised to 325 K for a 100 ps NVT simulation. Finally, a 100 ps NPT simulation was conducted, relaxing the system into an isotropic Parrinello-Rahman barostat [Parrinello et al, 1981; Nosé et al, 1983] set to 1.0 bar of pressure in all directions and a pressure coupling time constant (τP) of 2.0 ps. The production MD of 10 ns was performed with the velocity rescale thermostat and Parrinello-Rahman barostat, as well as the LINCS and PME treatments as described. Snapshots of the trajectory were taken in every 2 ps.

Accelrys DS Studio was used for structural alignments and visualization. Graphs were prepared in Microsoft excel.
3.2 RESULTS AND DISCUSSIONS

When the activity of L-LDH was studied at different temperatures, no loss of activity was observed below 50°C; complete deactivation occurred transiently at 52°C. Therefore, the holdase property of IbpA, IbpB or (IbpA+IbpB) was studied with respect to the inhibition of deactivation of LDH at 52°C. The results are shown in fig. 3.1A. In the figure curve-a represents the activity kinetics of native LDH at 30°C; within 15-20 minutes, the substrate was almost used up i.e., NADH was converted to NAD+. Curve-b signifies complete inactivation of LDH at 52°C. When the inactivation of LDH at 52°C was allowed in the presence of IbpA, curve-c appeared, indicating some activity of LDH. This restoration of LDH activity was surely due to the holding chaperone activity of IbpA. Curves- d & e show the deactivation kinetics of LDH at 52°C in the presence of IbpB and (IbpA+IbpB) respectively. Comparison of the curves- c, d & e clearly implied that the holdase activity of IbpB was greater than that of IbpA and the activity of IbpA and IbpB in combination was further higher than the individual activity of IbpA or IbpB. Defining the relative chaperone activity as:

\[
\text{\% inhibition of deactivation} = \frac{[(A_{340})_{\text{LDH at 52°C}} - (A_{340})_{\text{LDH + IbpA/B/A+B at 52°C}}] \text{at 20 min}}{[(A_{340})_{\text{LDH at 52°C}} - (A_{340})_{\text{LDH at 30°C}}] \text{at 20 min}} \times 100
\]

[since, after 20 min, the substrate NADH was almost used up by the native LDH]

the holdase activity of IbpA, IbpB and (IbpA+IbpB) on deactivating LDH at 52°C were calculated to be about 27, 48 and 57% respectively.
Figure 3.1 Spectrophotometric representation of the holding chaperone activity of IbpA/B/AB. A. Suppression of inactivation of LDH, kept for different times at 52°C, in the presence of IbpA, IbpB and IbpAB (above). B. Suppression of aggregation of LDH, kept for different times at 60°C, in the presence of IbpA, IbpB and IbpAB (below).
With deactivation, no aggregation of LDH took place at 52°C or at any temperature below 60°C; aggregation phenomenon occurred transiently at 60°C. Therefore, the holdase property of IbpA/IbpB/(IbpA+IbpB) with respect to the inhibition of aggregation of L-LDH at 60°C was investigated. The results are shown in fig. 3.1B. In the figure curve-a represents the aggregation of denatured LDH with time at 60°C; complete aggregation took place within 300 sec of shifting the temperature to 60°C. When the aggregation of LDH at 60°C was allowed in the presence of IbpA, IbpB and (IbpA+IbpB) separately, the aggregation kinetics looked like curves- b, c & d respectively, which clearly showed some inhibition in aggregation of LDH due to the holding chaperone activity of IbpA/B/A+B. Fig. 3.1B also shows that the suppressing power of aggregation (or in other words, holding chaperone activity against aggregation) of IbpB was more than that of IbpA and the combined action of IbpA and IbpB was stronger than that of the IbpA or IbpB alone. Defining the relative chaperone activity as:

\[
\text{% inhibition of aggregation} = \frac{(O.D\ 360)_{\text{LDH at 60}^\circ C} - (O.D\ 360)_{\text{LDH + IbpA/B/A+B at 60}^\circ C}}{(O.D\ 360)_{\text{LDH at 60}^\circ C} - (O.D\ 360)_{\text{LDH at 30}^\circ C}} \times 100
\]

[since, within 10 min of shifting the temperature at 60°C, the value of (O.D)360nm reached plateau region]

the holdase activity of IbpA, IbpB and (IbpA+IbpB) on aggregating LDH was found to be about 22, 30 and 33% respectively. The percentage of holdase activity inhibiting deactivation at 52°C was higher than that inhibiting aggregation at 60°C; this was perhaps due to the comparatively lower activity of the heat-shock chaperones IbpA / IbpB at 60°C than at 52°C. In support of this it can be mentioned that when the inhibition of aggregation of DTT-denatured insulin in the presence of IbpA / IbpB was studied at different temperatures viz., 40°C, 50°C and 60°C, the inhibition was found to be the maximum at 50°C [Fig. 3.2].
Question might be raised whether the effect of IbpA / IbpB was an artifact. For this, inactivation and aggregation experiments of LDH were performed by adding 0.5 mg/ml BSA, in place of IbpA or IbpB, as control. No suppression of inactivation or aggregation by BSA was observed, rejecting the validity of the question.

The above results signified that there was a threshold thermal energy equivalent to 52°C above which complete deactivation of LDH occurred, possibly due to a conformational alteration at its active site and there was a threshold thermal energy equivalent to 60°C above which LDH was aggregated, possibly due to gross alteration in conformation of the whole protein. Following results of molecular dynamics simulation on LDH corroborated the concept of conformational alteration at active site at 52°C. The active site [AC1 that represents the binding site for 1, 4 dihydro NAD] geometry of the simulated L-LDH at 325 K (i.e. 52°C) was compared with its crystallographic structure (PDB code 3H3F) by calculating the shortest distance between the atomic planes of the approaching active site residues over the period of simulation. From the simulation result,
as shown in fig. 3.3 A, it was evident that at the deactivation temperature (52°C), the relative distances between the catalytic site residues (viz., 192H-137N, 137N-251I, 251I-30V, 30V-135V, 30V-94T, 28G-51D, 96G-115I, 115I-52V) of LDH increased in each case; most significantly between 28G-51D. These changes in turn distorted the active site geometry and prevented NADH binding. The three dimensional view of the change of the coordinates of the aforementioned amino acid pairs has been presented in fig. 3.3B, which clearly shows alteration in active site of LDH at 52°C. On the other hand, the simulated value of the radius of gyration of LDH at 325 K (3.15 ± 0.005 nm) was almost remained unchanged compared to its experimental value (3.12 nm) obtained from PDB. Of the 10.0 ns of simulation, the insignificant increase in the radius of gyration (3.12 nm → 3.15 nm) was found to occur within 2.0 ns of simulation, above which no further change took place (fig. 3.3C), indicating that the active site denaturation of LDH at 52°C was a fast process and later on no other denaturation occurred. Thus the MD simulation clearly implied that due to the heat-mediated denaturation of LDH at 52°C, conformational alteration occurred at its active site without any significant change in the radius of gyration i.e. the size of the protein.
Spectrofluorimetric study showed that when L-LDH was excited at 280 nm, it had fluorescence with emission maxima at 350 nm. This was due to the presence of six tryptophan residues per monomer of the tetrameric LDH. On the other hand IbpA or IbpB had no significant intrinsic fluorescence (IbpB had two tryptophan residues, whereas IbpA had none, UniProt accession number P0C058 and P0C054 respectively) [www.uniprot.org,]. The fluorescence of native LDH at 30°C (curve-a, fig. 3.3A) quenched heavily at 52°C (curve-c, fig. 3.3A) and 60°C (curve-d, fig. 3.3A); the extent of quenching at
60°C was more than that at 52°C. The fluorescence spectra supplemented our spectrophotometric finding that the extent of denaturation at 52°C was less than that at 60°C. Fig. 3.4A also shows that the fluorescence of native LDH at 30°C did not alter much by the presence of IbpA+IbpB (curve-b, fig. 3.4A), implying that the small heat-shock chaperones IbpA/B could not bind native LDH at ambient temperature. At both the temperatures 52 and 60°C, the extent of fluorescence quenching of LDH in the presence of IbpA was found to be more (curves-a & b, fig. 3.4B respectively) than that of LDH alone (curves-c & d, fig. 3.4A respectively). But in principle, due to IbpA-mediated inhibition of deactivation or aggregation of LDH, the quenching of LDH fluorescence should be less in presence of IbpA than in absence of it. Therefore, the more, instead of the less, quenching by the presence of IbpA might be due to the binding of the small heat-shock chaperone IbpA on the denaturing LDH and the increase of quenching due to binding of IbpA perhaps superseded the decrease of quenching due to suppression of deactivation or aggregation. Furthermore, no occurrence of binding between LDH and IbpA at ambient temperature (30°C) and the occurrence of binding at 52/60°C were justified because of the chaperonic property of IbpA under heat-stress. Comparison of the curves-a & b of the figures 3.4C & 3.4D with those of figure 3.4B clearly indicated that at either of the temperatures 52°C and 60°C, the quenching of LDH fluorescence in the presence of (IbpA+IbpB) > that in presence of IbpB > that in presence of IbpA; this result signified that on denatured or aggregated LDH, the binding of IbpB was more than that of IbpA and in combination IbpA and IbpB had stronger binding.
Figure 3.4 Fluorescence spectra of L-LDH. A: LDH alone; B: LDH + IbpA; C: LDH + IbpB; D: LDH + IbpA + IbpB
Fig. 3.5 shows the results of the viscometric study. The viscosity of the native L-LDH at 30\(^0\)C was measured to be 0.92 (± 0.05) cP (centi Poise). At 52\(^0\)C, the viscosity increased slightly to 0.97 (±0.095) cP and at 60\(^0\)C, it increased considerably to 1.53 (± 0.029) cP. Change in viscosity of a protein solution is generally caused by the change in size and or shape of the protein molecules [Tu et al, 2005, Gregorová et al, 2009]. With reference to the shape of a macromolecule, there is a physical parameter called Perrin factor or aspect ratio (the ratio of oblate to prolate of an ellipsoid); increase/decrease of this ratio signifies increase/decrease of viscosity. In our case, the Perrin factor of LDH, as determined by the ‘protein utility tool’ of the particle size analyzer (Malvern, NanoZS), was found to have no significant change at 52\(^0\)C compared to 30\(^0\)C, whereas it increased enormously at 60\(^0\)C (data not shown). It, therefore, seemed that the partial denaturation of LDH at 52\(^0\)C possibly caused little change of its shape/size and so, an insignificant change of viscosity (0.92 cP → 0.97 cP) took place. But the gross denaturation and consequent aggregation at 60\(^0\)C caused such a large increase in shape/size of LDH aggregate, that was reflected in the high values of Perrin factor and viscosity. Since, the viscosity of LDH at 52\(^0\)C did not alter considerably from its value at 30\(^0\)C, therefore, to understand the holdase property of IbpA/B/A+B on denaturing LDH, viscometric study was performed only at 60\(^0\)C. It should be mentioned here that each of IbpA/B/A+B had an average viscosity of about 1.0 cP at both 30\(^0\) and 60\(^0\)C i.e., no denaturation and so, no change in size of IbpA/B/AB had occurred at 60\(^0\)C. Moreover, the viscosity of (LDH + IbpA/B/A+B) at 30\(^0\)C was also about 1.0 cP (data not shown). Therefore, LDH alone, each of IbpA/B/A+B and each of (LDH + IbpA/B/A+B), all had the viscosity close to 1.0 cP at 30\(^0\)C. However, the viscosity of LDH alone at 60\(^0\)C (1.53 cP) reduced to 1.36 (± 0.051), 1.23 (± 0.045) and 1.05 (±0.039) cP, when the viscosity of LDH was measured at 60\(^0\)C in the presence of IbpA, IbpB and (IbpA + IbpB) respectively (fig. 3.5). The low value of viscosity signified that smaller aggregates were formed in the presence of IbpA/B/A+B, or in other words, inhibition in aggregation of LDH by the holdase property of IbpA/B/A+B. The holdase activity of IbpA, IbpB and (IbpA+IbpB), as calculated from the values of viscosity change, was found to be 28, 46 and 75% respectively and this was qualitatively similar to
spectrophotometric study i.e., holding chaperone activity of IbpA < that of IbpB < that of (IbpA+IbpB).

The result of the above viscometric study was in strong conformity with the result of the dynamic light scattering experiment. Just like the changes in viscosity, the size of native LDH at 30°C [7.5 nm (± 0.43)] increased slightly to 8.1 (± 0.36) nm at 52°C, and increased considerably to 3330 nm at 60°C (fig. 3.6). This result clearly implied that although the LDH was completely inactivated at 52°C, however, its size remained almost unchanged; on the other hand, the aggregation of denatured LDH at 60°C caused enormous increase in size of LDH. It should be mentioned here that at 30°C, 52°C and 60°C, the sizes of IbpA were measured to be 8.5, 10.5 and 16.7 nm respectively and that of IbpB were 7.9, 10.4 and 18.3 nm respectively, which indicated that no significant change in the sizes of the heat-shock chaperone proteins IbpA and IbpB took place when they were shifted from ambient to 52°C or 60°C. The strength of holdase property of IbpA/B/A+B was apparent

**Figure 3.5 Viscosity of L-LDH at different experimental conditions.** (A) native LDH at 30°C; (B) LDH at 52°C; (C)LDH at 60°C; (D) (LDH + IbpA) at 60°C; (E) LDH + IbpB at 60°C and (F) (LDH + IbpAB) at 60°C.
from the observations that the size of LDH was 3330 (±270.5), 1301 (±35.5), 1137 (±14.5) and 1005 (±34.97) nm when it was allowed to incubate alone and in presence of IbpA, IbpB and (IbpA + IbpB) respectively at 60°C (fig. 3.6). The holdase activity of IbpA, IbpB and (IbpA+IbpB), as calculated from the values of size change, was found to be 61, 66 and 70% respectively. Therefore, it was further established that the holdase activity of IbpB was higher than that of IbpA and in combination, they had more intense role. It is desirable to mention here that at 30°C, both native LDH (tetramers of molecular weight 150 kDa [Świderek et al, 2009]) and IbpA/IbpB (multimers of molecular weight 2-3 MDa [Kitagawa et al, 2002; Shearstone et al, 1999]) were found to have nearly same DLS sizes of 7.5 nm and 8.5/10.5 nm respectively and at 52°C, both IbpA (monomers of molecular weight 15.7 kDa [Kitagawa et al, 2002; Shearstone et al, 1999]) and IbpB (oligomers of molecular weight 650-700 kDa [Kitagawa et al, 2002; Shearstone et al, 1999]) had similar sizes of 10.5 and 10.4 nm respectively; these results signified that the size/shape of active proteins had no linear dependency on their molecular weights, which was also evident from the other DLS results that showed globular proteins of molecular weights ranging from 17.6 kDa to 1.72 MDa had sizes within 2.4 to 12.3 nm [Erickson et al, 2009].

![Figure 3.6 Size of L-LDH at different experimental conditions. (A) native LDH at 30°C; (B) LDH at 52°C; (C)LDH at 60°C; (D) (LDH + IbpA) at 60°C; (E) LDH + IbpB at 60°C and (F) (LDH + IbpAB) at 60°C.](image)
The holding chaperone activity of IbpA/B/A+B on the changes in the shape and size of aggregating L-LDH at $60^\circ$C were visible from the AFM images (fig. 3.7). The native LDH appeared to be ellipsoidal in shape (image A, fig. 3.7). The oblate size of LDH at $30^\circ$ and $52^\circ$C was measured to be 0.031 and 0.037 µm respectively (from the images A & B), which were nearly similar. However, the LDH appeared as large aggregates (keeping ellipsoidal shape) of oblate size 7.843 µm at $60^\circ$C (image C). In the presence of IbpA, IbpB and (IbpA+IbpB), size of LDH aggregate at $60^\circ$C was found to be gradually smaller i.e., 1.120 µm, 0.206 µm and 0.120 µm respectively (images D, E & F, fig. 3.7). Smaller aggregates signified lesser aggregation of LDH, when it was allowed to denature at $60^\circ$C in the presence of IbpA/B/A+B and the lesser aggregation surely implied the holdase property of IbpA/B/A+B. It might be questioned whether the images D, E & F represented LDH aggregates or IbpA, IbpB and (IbpA+IbpB) respectively. Since, the AFM samples, after fixing on the mica surface, were washed with imidazole, the histidine-containing IbpA and IbpB were removed away from the surface due to high binding affinity of the imidazole with histidine and so invalidating the question. Thus, the results of this atomic force microscopic study resembled qualitatively well with all the above findings.
CONCLUSION:

The ultimate revelations from the whole study were that the *E. coli* small heat-shock protein IbpA had less holding chaperone activity than IbpB on denaturing and aggregating protein and when they acted in combination, the effect was more profound i.e. the activity of (IbpA+IbpB) was higher than that of either IbpA or IbpB. This communication, together with the very few earlier reports, signified that the application of modern sensitive techniques such as micro-viscometry, dynamic light scattering and atomic force microscopy in the study of protein folding may reveal finer details about the said phenomenon.

Figure 3.7 AFM images of L-LDH at different experimental conditions. (A) native LDH at 30°C; (B) LDH at 52°C; (C) LDH at 60°C; (D) (LDH + IbpA) at 60°C; (E) LDH + IbpB at 60°C and (F) (LDH + IbpAB) at 60°C.