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Silkworm sHSP19.9 and sHSP20.8 with ATP on the thermal induced aggregation of Bovine Liver Catalase (BLC)

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Abstract: Adenosine Tri Phosphate (ATP) plays a significant role in the function of molecular chaperones of the large heat shock protein families. However, its role in the functions of chaperones of the small heat shock protein (sHSP) families is not understood very well. This paper reports the findings of a study on the role of ATP on the structure and function of the two important small heat shock proteins (sHSPs); sHSP19.9 and sHSP20.8. The study revealed that ATP at high concentration (10 mM) helps sHSP19.9 to show effective CLA against bovine liver catalase (BLC) aggregation at 60ºC rather than at low (3 mM) concentration. ATP induces the association of sHSPs with substrate proteins and stabilizes the sHSPs structure. But sHSP20.8 shows opposite; the potential performance was observed at low concentration of AT (below 3 mM), which messaged that the association process is low affinity in nature. So the ATP-induced association of the both sHSPs with substrate enhanced its aggregation prevention ability and also enhanced the refolding yield of BLC from the unfolded state.

Key words: ATP, CLA, Silkworm, Small heat shock protein, sHSP19.9 and sHSP20.8.

Introduction

Molecular chaperones of the large heat shock protein families not only suppress protein unfolding and aggregation in response to stress but also actively participate in the refolding of denatured proteins *in vitro*, often is an ATP-dependent manner (Hartl, 1996; Gething and Sambrook, 1992). CLA has been described for sHSP19.9 and sHSP20.8 from silkworm in suppressing protein unfolding and aggregation in response to thermal or chemical stress (Hossain *et al*., 2010). At high temperature stress, sHSP could prevent the accumulation of heat denatured protein aggregates and/or facilitate protein reactivation following stress (Parsell *et al.,* 1994). To functionally characterize sHSP19.9 and sHSP20.8 as a molecular chaperone, their effect on the unfolding and refolding of bovine liver catalase (BLC) in the presence and absence of ATP were studied *in vitro*. BLC was chosen as a model protein for the characterization of unfolding and refolding reactions because it is a ubiquitous enzyme present in aerobic organisms. It catalyzes the disproportionation of H_2O_2 to molecular oxygen and water. Apart from the natural substrate, H_2O, some alkyl hydroperoxides can act as BLC substrate as well (Karra-Chaabouni *et al.,* 2002). BLC is readily aggregated and inactivated at 60ºC (Hook and Harding, 1997).

ATP is an abundant phosphorous metabolite in lenses from many species at concentrations as high as 6.7 mM (Greiner, 1985; Greiner *et al.*, 1981; Pirie, 1962), which is among the highest levels of any cell in the body (Klethi and Mandel, 1965). High concentrations of ATP (4–8 mM) are found in skeletal muscle (Burt *et al*.,1976), in which high levels of αβ-crystallin are expressed (Bennardini *et al*., 1992). The activity of GroEL and other chaperones on unfolding and refolding of proteins has been characterized at high concentrations (3.5 mM) of ATP (Jakob *et al*., 1993; Buchner *et al*., 1991; Ayling and Baneyx 1996; Skowyra, 1990; Frydman, 1994; Martin, 1993), which may reflect a functional relationship between high concentrations of ATP and chaperone activity in all cells. The functional experiments with sHSP19.9 and sHSP20.8 in this report used ATP at a concentration of 3.5 mM. Not only this concentration comparable directly to that used in functional *in vitro* studies published for well characterized chaperones (Jakob *et al.,* 1993; Buchner *et al.*, 1991; Ayling and Baneyx 1996; Skowyra, 1990; Frydman, 1994; Martin, 1993), but this is a concentration that is present *in vivo* in cells that express αβ-crystallin (Pirie, 1962; Greiner, 1985; Greiner *et al.*, 1981; Klethi and Mandel, 1965; Burt *et al*.,1976; Bennardini *et al.*, 1992) that shares sequence and functional similarities with small heat shock proteins (Muchowski and Clark, 1998). The aim of the work was to find out the function of sHSP19.9 and sHSP20.8 as an ATP-enhanced molecular chaperone not only in suppression of the unfolding and aggregation of BLC but also in mediating the proper refolding of BLC to a fully functional conformation.

Materials and Methods

Chemicals used

All other reagents used were of analytical grade without further purification.

Buffer preparation: Unless otherwise noted, the used buffers were 50 mM sodium phosphate buffer (pH 8.0) containing 0.1 M NaCl (buffer A), containing 0.3 M NaCl (buffer B), and 20 mM HEPES buffer (pH 7.7) containing 10 mM NaCl (buffer C).

Over production and Purification of sHSPs: Overproduction of sHSPs by *E. Coli* cells and purification of the overproduced sHSPs were done as described by Hossain *et al.* (2010). Thus N-terminal His-tagged recombinant sHSPs were prepared. Concentration of sHSP19.9 and sHSP20.8 were calculated using molar extinction coefficients ($\varepsilon_{280\ nm}$); 19940 cm^{-1} M^{-1} and 22585 cm^{-1} M^{-1} at 280 nm respectively (Pace *et al.,* 1995).

Preparation of Bovine liver catalase (BLC): BLC was purchased from Sigma (Tokyo, Japan). BLC solution was freshly prepared with buffer C and used without further purification. Concentration of the BLC was determined by spectrophotometry, using $\varepsilon_{280\ nm}$ = 93.706 $mM^{-1}cm^{-1}$ (Decker, 1977).

Preparation of ATP: ATP was purchased from Oriental Co. Ltd. (Tokyo, Japan) and always freshly prepared ATP was used. ATP was prepared with the above mentioned buffer according to the necessary and concentration of ATP was calculated using $\varepsilon_{280\ nm}$; 15400 cm^{-1} M^{-1} at 260 nm (Boehmer and Emmerson, 1992).

Thermal aggregation assay of BLC: Concentration- and time- dependent thermal aggregation of BLC was monitored at 60ºC by spectrophotometer (Shimadzu UV-2400) method in the presence of buffer C. Different concentration of BLC ranged from 5.20 to 13.0 µM was used to monitor the aggregation up to 30 min with 5 min intervals. Different buffers were also used where necessary to observe the mode of aggregation at 360 nm.

CLA of sHSP19.9 against BLC aggregation in the presence of ATP: sHSP19.9 was observed to be aggregated in the presence of low NaCl concentration buffer C and the aggregation was concentration- and time-dependent, which was monitored spectrophotometrically at 360 nm using a concentration series ranged from 1.86 to 3.71 µM. At this situation it was unable to show CLA, which was the clue to find out if there any positive effect of ATP in the lower ionic strength buffer. The effect of ATP on the CLA of sHSP19.9 against the thermal induced aggregation of BLC was investigated with 1:1 (5.0 µM each of sHSP19.9 and BLC) molar concentration ratio and different concentration of ATP; 0 (control), 2, 2.5, 3.0, 3.5 and 4.0 mM.

On the other hand such kind of unusual aggregation of sHSP19.9 was successfully suppressed in the presence of higher ionic strength buffer. Moreover in this condition, it can also suppress the thermally-induced aggregation of other non-native protein like BLC. So, different concentration ratio 1:0 (BLC 5.0 µM), 1:0.1 , 1:0.2 , 1:0.5, 1:1 and 1:2 for sHSP19.9 and BLC were used to observe the performance by using the above mentioned method. In the presence of above mentioned reaction buffer, the CLA of sHSP19.9 was monitored against BLC aggregation with the same molar ratios and different concentrations of ATP; 0 (control), 2, 3.5, 5.0 and 10.0 mM. The effectiveness of ATP for both cases was monitored at 360 nm by spectrophotometer (Shimadzu UV-2400) method.

CLA of sHSP20.8 in the presence of ATP: Using the similar method mentioned above, the effect of ATP on the CLA of sHSP20.8 was observed at 360 nm. Buffer C (control) and 0.1 M HEPES buffer (pH 7.7) containing 0.1 M NaCl were used. For both the cases, sHSP20.8 and BLC at 1:1 (5.0 µM each) molar ratio was used in the presence of different concentration of ATP ranged from 0 to 5.0 mM.

Results

Thermal aggregation of BLC and sHSP19.9 and CLA of sHSP19.9 against BLC aggregation: BLC is a ubiquitous enzyme was used as non-native target protein to observe the heat stress time and concentration dependent changes. To observe the aggregation very intensively the absorbance was monitored on second basis of time of incubation. All of the concentrations and incubation time showed the alike absorbance at the starting point (0). With the increase of incubation time and concentration of BLC the absorbance at 360 nm was observed to be increased (Fig. 1) due to the formation of BLC turbid in the reaction medium and it was happened due to the aggregation of BLC, which absorbace more light. However, sHSP19.9 with low ionic strength buffer behaved the same as BLC at 60°C. With the increase of incubation time and concentration, its absorbance at 360 nm was increased and it was due to the aggregaation of sHSP19.9 (Fig. 2).

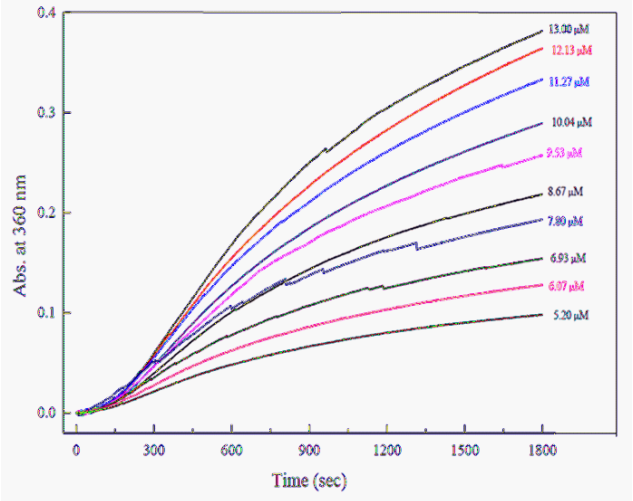

Fig. 1. Time and concentration-dependent thermal aggregation of BLC at 60ºC in the presence of buffer C (lower ionic strength buffer) by spectrophotometer (Shimadzu UV-2400) method. Different concentration series ranges from 5.20 to 13.0 µM and time series from 0 to 30 min was used.

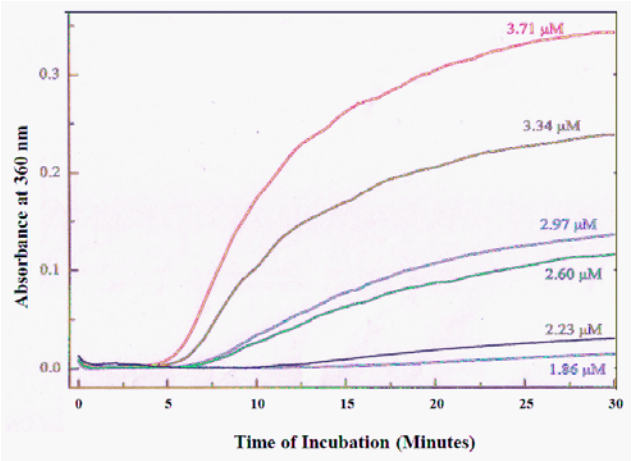

Fig. 2. Thermally-induced aggregation of sHSP19.9 at 60ºC in the presence of lower ionic strength buffer (buffer C) by spectrophotometer (Shimadzu UV-2400) method. A series of concentration ranged from 1.86 to 3.71 µM was used besides 30 minutes incubation with 5 minutes intervals.

But in the presence of higher ionic strength buffer sHSP19.9 was capable to tolerate the heat induced changes, whereas BLC was vulnerable. At the same condition sHSP19.9 could effectively suppress the aggregation of BLC at 60°C (Fig. 3). Different concentration ratio for BLC and sHSP19.9 was used and 1:2 was observed to the best one as at this ratio sHSP19.9 could suppress the aggregation of BLC completely. Using the similar condition the effect of ATP on the enhancement of CLA of silkworm sHSPs was investigated and discussed below.

Effect of ATP on CLA of sHSP19.9: The effect of nucleotide like ATP on the CLA of sHSP19.9 was examined. According to the published data obtained with

αβ-crystallin (Muchowski and clark, 1998) both above and below 3.5 mM were selected. Two different buffers; buffer C and 0.1 M HEPES buffer (pH 7.7) containing 0.1 M NaCl were used. In Fig. 3, as the molar ratio of BLC to sHSP19.9 at 1:1 was observed to be satisfactory effective to suppress almost complete aggregation, so the molar ratio was fixed at 1:1. In buffer C (Fig. 4) lower concentration of ATP; 2.0 and 2.5 mM had no any effect on the CLA of sHSP19.9, when compared to control. But 3.0 mM and above concentration of ATP showed negative effect as the aggregation was enhanced.

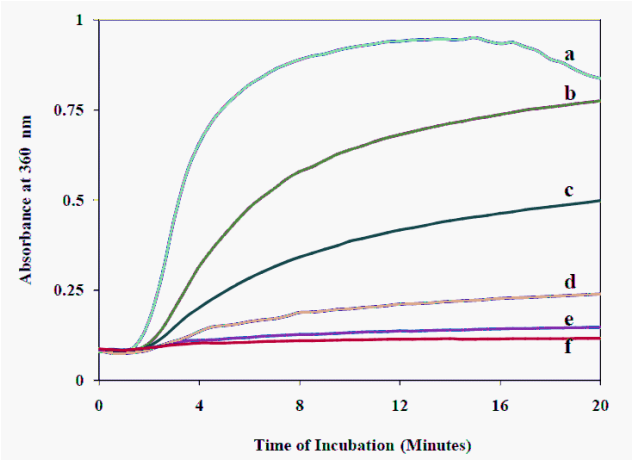

Fig. 3. CLA of sHSP19.9 against BLC aggregation at 60ºC in the presence of higher ionic strength buffers (buffer A).Different molar ratio such as 1:0 (a: BLC 5.0 µM), 1:0.1 (b), 1:0.2 (c), 1:0.5 (d), 1:1 (e) and 1:2 (f) were used to observe the activity by spectrophotometer (Shimadzu UV-2400) method.

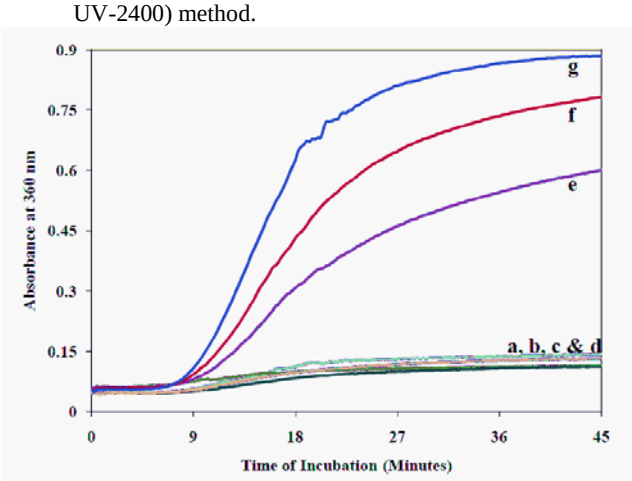

Fig. 4. CLA of sHSP19.9 (5.0 µM) against BLC (control; 5.0 µM) aggregation in the presence of ATP with buffer C. The effects was monitored by by spectrophotometer (Shimadzu UV-2400) method with 0 (b), 2.0 (c), 2.5 (d), 3.0 (e), 3.5 (f), and 4.0 mM (g) concentration of ATP with control (a; 5.0 µM BLC only).

Concentration-dependent performance for ATP was observed (Fig. 5) upon usage higher ionic-strength containing second buffer. As the high concentration of NaCl and HEPES stabilize the sHSP19.9 at elevated temperature so that in the presence of 0.1 M HEPES buffer (pH 7.7) containing 0.1 M NaCl buffer, the apparent suppression of BLC aggregation by sHSP19.9 was increased with increasing concentration of ATP.

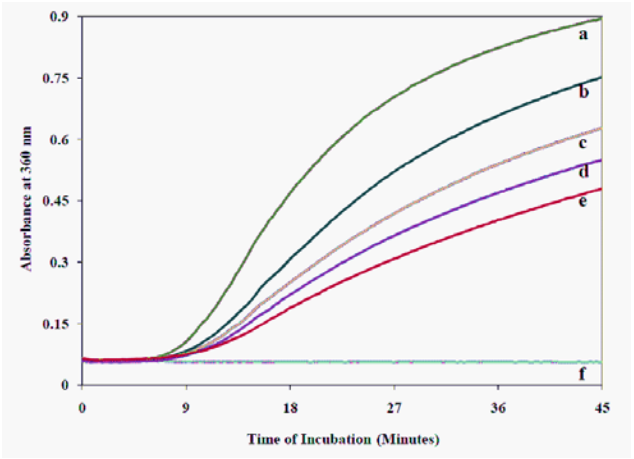

Fig. 5. CLA of sHSP19.9 (5.0 µM) against BLC (control; 5.0 µM) aggregation in the presence of ATP with 0.1 M HEPES buffer pH 7.7 containing 0.1 M NaCl. The effects was monitored by by spectrophotometer (Shimadzu UV-2400) method with with 0 (a) , 2.0 (b), 3.5 (c), 5.0 (d) and 10 mM (e) ATP with control (f; 5.0 µM BLC only).

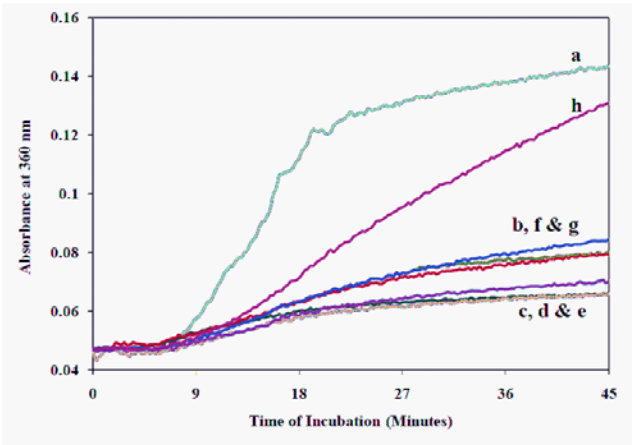

Fig. 6. sHSP20.8 (5.0 µM) with ATP against BLC (a: 5.0 µM BLC) aggregation in the presence of buffer C. ATP of different concentration such as 0 (b: control), 2.0 (c), 2.5 (d), 3.0 (e), 3.5 (f), 4.0 (g) and 5.0 (h) mM was used. The CLA was monitored by by spectrophotometer (Shimadzu UV-2400) method.

Effect of ATP on the CLA of sHSP20.8: Effect of ATP on the CLA of sHSP20.8 was examined in the presence of lower ionic strength buffer C. It was observed that 3.5 mM and 4.0 mM of ATP affect the CLA of sHSP20.8 but ineffective as their performances are similar to the control (0 mM ATP). ATP at 5.0 mM promoted the unfolding of BLC and accelerated its aggregation, where sHSP20.8 was observed to be inefficient to show its CLA (Fig. 6) because the high concentration of ATP lowers the affinity for substrate for sHSP20.8.

CLA of sHSP20.8 in the presence of ATP was also examined using higher ionic-strength buffer; 0.1 M HEPES (pH 7.7) containing 0.1 M NaCl (Fig. 7). Besides control (0 mM ATP), wide ranges of ATP were used. But

their performances were similar to the control, which messaged that ATP had no effects on the CLA of sHSP20.8 at higher ionic condition. Smýkal *et al*. (2000) observed the similar findings that lower concentration had no effects and the higher concentrations had the negetive effects on the CLA of tobacco HSP18 against citrate synthase aggregation.

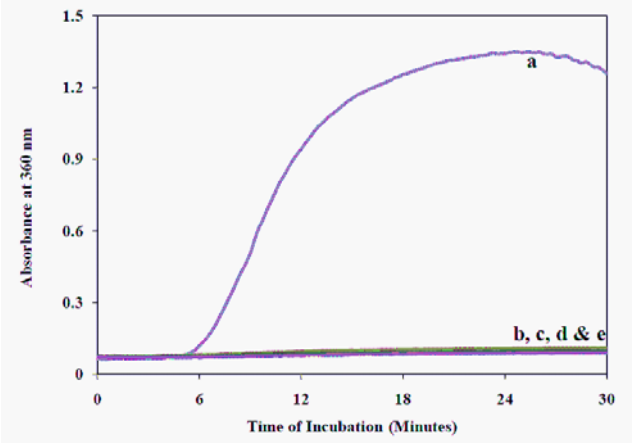

Fig. 7. CLA of sHSP20.8 (5.0 µM) against BLC (a: 5.0 µM BLC) aggregation in the presence of 0.1 M HEPES buffer (pH 7.7) containing 0.1 M NaCl buffer. A concentration series of ATP such as 0 (b: control), 2.0 (c), 3.5 (d), and 5.0 (e) mM was used. The activity was monitored by spectrophotometer (Shimadzu UV-2400) at 360 nm.

Discussions

The presence of ATP enhanced the reactivation yield in some cases (Muchowski and Clark, 1998; Wang and Spector, 2001). The role of ATP in such experiments is not fully understood because diversified effects of ATP were evident for the studied sHSPs. On the contrary, the role of ATP is much better understood for the refolding of enzymes mediated by the chaperones GroEL, DnaK, etc. (Hartl, 1996). Taking clue from such a mechanism, a similar role of ATP has also been envisaged for the chaperone activity of silkworm sHSP19.9 and sHSP20.8. Although the role of ATP for the chaperone-like like activity of sHSPs has also been debated in the literature but Muchowski and Clark (1998) reported that the refolding yield of substrate protein reduced when ATP was replaced by nonhydrolyzable ATPase. However, Rawat and Rao (1998) reported that ATP hydrolysis is not required. Moreover, according to Hartl, (1996) molecular chaperones are of the large HSP families not only suppress protein unfolding and aggregation in response to stress but also actively participate in the refolding of denatured proteins *in vitro*, often in an ATP-dependent manner, which was supported by Gething, & Sambrook, (1992). So the exact effect of ATP on the CLA of sHSPs is not well understood. For silkworm sHSP28.8 and sHSP19.9, ATP showed sometimes significant and negative effect on the CLA. The negative effect for sHSP19.9 was observed in the presence of low ionic strength (buffer C), which matched with the performance of tobacco HSP18 (Smýkal e*t al*., 2000) and HSP90 (Jakob *et al.,* 1995), which are also inhibited by ATP to show CLA against citrate synthase. But significant positive effect of ATP to influence the CLA of sHSP19.9 was found in the presence of high ionic strength buffer (0.1 M HEPES buffer (pH 7.7) containing 0.1 M NaCl), where the concentration-dependence partial suppressive activity of sHSPs was observed against BLC aggregation at 60˚ C temperature. It is in aggreement with the findings of Muchowski and Clark (1998) for human αβ crystallin, whereas sHSP19.9 was obviously performed as concentration-dependent but complete suppression was not observed although higher concentration of ATP was used.

The dissociation by ATP is often activated by its hydrolysis carried out by the fairly well known ATPase activity of the above-mentioned chaperones. Moreover, ATP weakens the interactions between chaperone and substrate and thus helps the latter to refold (Wang and Spector, 2000; Wang and Spector, 2001). So the positive effect for sHSP20.8 was not observed. Although lower concentration of ATP did not affect the interaction but above 3.5 mM concentration brought insignificant negative result. Moreover, 5.0 mM and above might cause the damage of interaction and so sHSP20.8 was observed to be disable to suppress the aggregation of BLC. On the other hand, at higher ionic concentration such interaction was not evident, where the higher NaCl concentration might strengthen the interaction of sHSP20.8 and BLC. So that all of the possible combination and concentration of ATP showed the identical results. As the refolding of BLC was not observed due to the stable interaction, so insignificant effect of sHSP20.8 was observed to show the CLA even in the presence of ATP.

Silkworm sHSP; sHSP19.9 and sHSP20.8 function as molecular chaperone in suppression of the unfolding and irreversible aggregation of BLC. In the presence of higher ionic strength buffer not buffer C, the thermally induced aggregation of sHSP19.9 was suppressed and in this condition the CLA was also enhanced by ATP. Such activity for sHSP20.8 was not revealed due to the strong interaction between sHSP20.8 and BLC, which help BLC to be remained unfolding condition.

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