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HSP-1/2, a major protein of equine seminal plasma, exhibits chaperone-like activity

Rajeshwer Singh Sankhala [a,1], C. Sudheer Kumar [a,1], Bhanu Pratap Singh [a], A. Arangasamy [b], Musti J. Swamy [a,*]

[a] School of Chemistry, University of Hyderabad, Hyderabad 500 046, India
[b] Equine Production Campus, National Research Centre on Equines, Jorbeer, Bikaner 334 001, Rajasthan, India

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ABSTRACT

The major bovine seminal plasma protein, PDC-109 exhibits chaperone-like activity (CLA) against a variety of target proteins. The present studies show that the homologous protein from equine seminal plasma, HSP-1/2 also exhibits CLA and inhibits the thermal aggregation of target proteins such as lactate dehydrogenase, and DTT-induced aggregation of insulin in a concentration-dependent manner. Phosphorylcholine binding inhibited the CLA of HSP-1/2, suggesting that aggregation state of the protein is important for this activity. These results demonstrate that HSP-1/2 functions as a molecular chaperone *in vitro*, and suggest that it may protect other proteins of equine seminal plasma from unfolding/misfolding or aggregation. These results suggest that homologous proteins from the seminal plasma of other mammals also exhibit CLA, which will be physiologically relevant.

1. Introduction

In mammals, seminal plasma carries the sperm cells from the male testes to the female uterus, where sperm–egg fusion takes place, which results in fertilization [1,2]. In the bovine seminal plasma, a group of acidic proteins are known to be involved in various stages of fertilization such as establishment of oviductal reservoir, sperm capacitation and sperm-zona pellucida interaction [3–5] and similar mechanisms seem to exist in many other mammals. Based on the structural characteristics, most of these proteins have been classified as fibronectin type-II (FnII) proteins, cysteine-rich secretory proteins and spermadhesins [6]. Among the FnII proteins, the major protein of bovine seminal plasma, PDC-109, has been studied in great detail [7–15]. PDC-109 binds specifically to phospholipids containing choline head group, such as phosphatidylcholine and sphingomyelin [3,12,16–20].

Proteins homologous to PDC-109 have also been identified in several other mammals [21]. Two such proteins in equine seminal plasma, HSP-1 and HSP-2, exhibit a high degree of homology and differ from each other in the extent of glycosylation and in the number of residues in the *N*-terminal segment [22–24]. HSP-1 is a polypeptide of 121 amino acids, with a 29 residue segment preceding the two FnII domains, whereas HSP-2 has a shorter (14 residue) *N*-terminal region. HSP-1 and HSP-2 share ~60% sequence similarity with PDC-109 [23,25]. These two homologous proteins could not be separated under non-denaturing conditions and their mixture is referred to as HSP-1/2. In previous work, the interaction of HSP-1/2 with phospholipid membranes was investigated using spin-label ESR and fluorescence spectroscopy [26]. The results obtained indicate that similar to PDC-109, HSP-1/2 show greater affinity for choline phospholipids [26]. The interaction of HSP-1/2 with phospholipid membranes and heparin appears to be physiologically significant in view of their involvement in sperm capacitation [6,24,26].

Recently, we have demonstrated that PDC-109 exhibits chaperone-like activity against a variety of target proteins by preventing their aggregation under stress conditions and that polydispersity and hydrophobicity are important for this activity [25,27]. Since HSP-1/2 shares significant homology with PDC-109 and exists in polydisperse oligomeric states [23,28], we considered that this protein may also exhibit CLA and carried out biochemical and biophysical studies to explore this possibility. The results obtained indicate that HSP-1/2 exhibits chaperone-like activity against a variety of substrate proteins. Presence of such chaperone proteins in the seminal plasma is of considerable physiological significance;

Abbreviations: ADH, alcohol dehydrogenase; AFM, atomic force microscopy; CD, circular dichroism; CLA, chaperone-like activity; DTT, 1,4-dithiothreitol; EDTA, ethylene diamine tetraacetic acid; ESR, electron spin resonance; FnII, fibronectin type-II; G6P, glucose-6-phosphate; G6PD, glucose-6-phosphate dehydrogenase; HSP-1/2, horse seminal plasma proteins 1 and 2; LDH, lactate dehydrogenase; NADP, nicotinamide adenine dinucleotide phosphate; PPC, *p*-aminophenyl phosphorylcholine; PrC, phosphorylcholine; TBS, 50 mM Tris buffer containing 0.15 M NaCl and 5 mM EDTA, pH 7.4.

* Corresponding author. Fax: +91 40 2301 2460.
E-mail addresses: mjssc@uohyd.ernet.in, mjswamy1@gmail.com (M.J. Swamy).
URL: http://www.202.41.85.161/~mjs/ (M.J. Swamy).

[1] These two authors contributed equally to this study.

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therefore establishment of their presence in other mammalian species including humans is of great interest.

2. Materials and methods

Details of materials and some experimental methods have been given in the supporting information.

2.1. Purification of HSP-1/2

HSP-1/2 was purified by affinity chromatography on heparin-agarose and PPC-agarose as described earlier [23,28] followed by reverse phase HPLC on a C-18 column (see Supplementary information for details). The purified protein was dialyzed extensively against 50 mM tris buffer, pH 7.4, containing 0.15 M NaCl and 5 mM EDTA (TBS) and stored at 4 °C. All experiments were carried out in the same buffer.

2.2. Circular dichroism spectroscopy

CD spectral studies were performed using a JASCO J-815 spectropolarimeter fitted with a thermostatted cell holder and a thermostatic water-bath at a scan speed of 50 nm/min. Far and near UV spectra were recorded using a 0.2 cm path length quartz cell at a HSP-1/2 concentration of ~0.12 mg/mL and 0.45 mg/mL, respectively. Each spectrum reported was the average of 10 consecutive scans from which buffer scans, recorded under the same conditions, were subtracted. Spectra were also obtained in the presence of 20 mM phosphorylcholine.

Thermal unfolding of HSP-1/2 was investigated by monitoring the CD spectral intensity of the protein (0.12 mg/mL) at 223 nm, while the temperature was increased from 25 to 80 °C at a scan rate of 1°/min. Effect of PrC binding on the thermal stability of HSP-1/2 was investigated by incubating the protein for ~30 min with a fixed concentration of PrC before the temperature scans were performed.

2.3. Computational modeling

The amino acid sequences of HSP-1, HSP-2 and PDC-109 were obtained from PubMed tool of NCBI (National Centre for Biotechnology Information) and submitted to I-TASSER server (http://www.zhang.bioinformatics.ku.edu/I-TASSER/) to build 3-dimensional structural models of the proteins. The crystal structure of PDC-109 (pdb code: 1h8p) was provided as a scaffold template. For each protein, the model with the lowest energy was used for further analysis and comparison.

2.4. Aggregation inhibition of substrate proteins by HSP-1/2

Chaperone activity was assayed as described previously [25] by monitoring the ability of HSP-1/2 to prevent heat-induced aggregation of alcohol dehydrogenase. ADH was incubated at 48 °C and its aggregation was monitored by recording light scattering at 360 nm as a function of time in a Perkin Elmer Lambda 35 UV/Visible spectrophotometer, which was also used for all other spectrophotometric measurements. A fixed concentration of HSP-1/2 in TBS was pre-incubated with 0.05 mg/ml of ADH for 5 min at room temperature and then experiments were performed as described above. ADH:HSP-1/2 (w/w) ratios of 1:0.2 and 1:0.5 were used. Aggregation profile for the native enzyme was taken as 100% and percent aggregation of other samples was calculated with respect to native enzyme. The effect of PrC binding was investigated by performing the aggregation assays after incubating HSP-1/2 for 10 min with different concentrations of PrC.

2.5. G6PD activity assay

G6PD activity was assayed by a spectrophotometric method essentially as described earlier for PDC-109 [25,29]. In this assay G6P is oxidized to 6-phospho-D-gluconate by G6PD with simultaneous reduction of NADP to NADPH. The reaction was initiated by addition of NADP to a mixture containing G6PD (0.25 μM), NADP (0.1 mM), G6P (5 mM) and 12 mM each of $MgCl_2$ and KCl, and increase in absorbance at 340 nm due to the reduction of NADP was monitored. To investigate the effect of HSP-1/2 on the thermal inactivation of the enzyme, 0.25 μM G6PD was incubated for 15 min in the absence or presence of 0.5 μM HSP-1/2 at 45 °C. Relative activities of various treated samples were normalized with respect to the native enzyme.

2.6. Insulin aggregation assay

Insulin aggregation assay was performed as described earlier [30]. In this assay, the ability of HSP-1/2 to prevent the aggregation of insulin B-chain caused by DTT is monitored. Briefly, 0.2 mg of insulin alone or upon incubation with different concentrations of HSP-1/2 was taken and 20 μL of freshly prepared DTT (1 M stock) was added and the volume was adjusted to 1 ml with TBS. The insulin:HSP-1/2 (w/w) ratios in different samples were 1:0.5, 1:1 and 1:1.5. The time course of aggregation was monitored at 360 nm for 1 h. Aggregation of native insulin was taken as 100% and percent aggregation of the remaining samples was calculated with respect to it.

2.7. Aggregation inhibition of LDH by HSP-1/2: AFM studies

LDH samples at a concentration of 75 μg/mL in TBS were incubated in the presence and absence of 1:1 (w/w) ratio of HSP-1/2 at 48 °C in a heating bath for 20 min and then transferred to an ice bath. A 25–50 μL aliquot of each sample was carefully deposited on a freshly cleaved mica sheet (1 cm × 1 cm) and allowed to dry for 20–30 min, rinsed with HPLC grade water, dried again and transferred to the AFM stage for imaging. Imaging was performed in semi-contact mode using a SOLVER PRO-M atomic force microscope (NT-MDT, Moscow, Russia) and the images obtained were analyzed using NOVA software, supplied by NTMDT as described earlier [25].

3. Results and discussion

3.1. Secondary and tertiary structure of HSP-1/2

Far-UV CD spectra of HSP-1/2 alone and in the presence of 20 mM PrC are shown in Fig. 1A. The spectrum of the protein alone (solid line) is characterized by a broad positive asymmetric band with maximum at 223 nm and a shoulder at ~210 nm. In the presence of PrC, the spectral intensity increases, although no major changes are observed in the shape of the spectrum (dashed line). The near-UV CD spectrum of HSP-1/2 contains two overlapping positive bands with maxima at ~282 nm and ~288 nm (Fig. 1B). These spectral features are similar to those of PDC-109 [10,31]. Similar to PDC-109, the positive band in the far UV CD spectrum of HSP-1/2 also could not be analyzed to obtain the secondary structure of the protein, due to the lack of a suitable reference dataset [31].

Thermal scans monitoring the CD spectral intensity at 223 nm, corresponding to the peak position of the far-UV spectrum of HSP-1/2, yielded a sigmoidal curve (Fig. 1C). For native HSP-1/2, the signal intensity exhibits the steepest decline at ~49 °C, indicating that the midpoint of the unfolding transition of the protein is ~49 °C

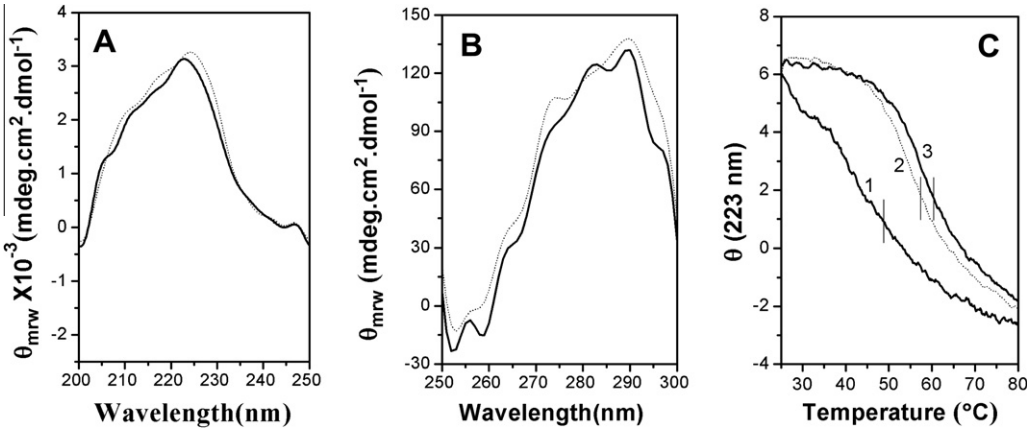

Fig. 1. Circular dichroism studies of HSP-1/2. (A) Far-UV CD spectra of HSP-1/2 alone (solid line) and in the presence of 20 mM PrC (dashed line). (B) Near-UV CD spectra of HSP-1/2 in TBS. (C) PrC induced thermal stability of HSP. Thermal scans of the protein (0.12 mg/mL) in the absence and presence of varying concentrations of PrC, at 223 nm are shown. The concentrations of PrC are: (1) 0 mM, (2) 10 mM and (3) 20 mM.

(curve 1, Fig. 1C). In the presence of 10 mM PrC the midpoint of the transition shifts to ~57 °C (curve 2), whereas increasing the PrC concentration to 20 mM shifts the transition midpoint to ~60 °C (curve 3). These results clearly show that PrC binding stabilizes the structure of the protein in a concentration dependent manner which is similar to that observed earlier with PDC-109 [31].

Since the near-UV CD spectra could not be interpreted to get information on the secondary structure of HSP-1/2, we employed computational methods to get 3-dimensional structural models of HSP-1, HSP-2 and PDC-109 using the I-TASSER server. Although the crystal structure of PDC-109 was known, it was necessary to generate a model from computational approach because the 23-residue *N*-terminal stretch of this protein was not seen in the crystal structure, possibly due to lack of order in the structure of this region [19]. The models obtained clearly show that the overall structures of HSP-1 and HSP-2 are very similar and that they closely resemble the structure of PDC-109 (Fig. S1). In order to compare them better, we obtained the secondary structures of the three proteins from the I-TASSER models. For PDC-109, the relative content of different secondary structures was also estimated from the crystal structure of the protein (pdb code: 1h8p). The results are given in a tabular form in the supporting information (Table S1). From this data it is clear that similar to PDC-109, both HSP-1 and HSP-2 contain very little α-helix and about 25% β-sheet, whereas nearly 70% of the residues are in loops and unordered structures. The structural plasticity of such largely unordered proteins is expected to facilitate their interaction with aggregation-prone target proteins in an effective manner, thus affording them protection under stress conditions.

3.2. Chaperone-like activity of HSP-1/2

3.2.1. Inhibition of heat-induced aggregation of target proteins by HSP-1/2

Results of turbidimetric studies aimed at investigating the effect of HSP-1/2 on the thermal aggregation of ADH are shown in Fig. 2A. When ADH was incubated at 48 °C, it is seen that turbidity of the sample increases rapidly with time, reaches a maximum and then levels off (curve 1). Presence of HSP-1/2 reduced the rate of this aggregation significantly in a concentration-dependent manner. An ADH to HSP-1/2 ratio (w/w) of 1:0.2 led to a considerable reduction in the rate of aggregation and at the end point of the assay the observed aggregation was ~40% as compared to that of the native enzyme (curve 2). The aggregation decreased further to 13% when the ADH to HSP-1/2 ratio was increased to 1:0.5 (curve 3). A bar diagram showing percent aggregation versus concentration of HSP-1/2 is given in Fig. 2B. HSP-1/2 exhibited similar chaperone-like activity against LDH also (Fig. S2). These results strongly suggest that HSP-1/2 protects target proteins from thermal denaturation, thus exhibiting CLA.

3.2.2. G6PD activity assay to probe CLA of HSP-1/2

To investigate whether HSP-1/2 can prevent the thermal denaturation of G6PD, we assayed the activity of this enzyme in the absence and in the presence of HSP. When incubated at 45 °C for 15 min G6PD lost about 50% of its activity (Fig. 2C, curve 2), whereas ~92% activity was retained when the enzyme was subjected to similar treatment in the presence of HSP-1/2 (curve 3). In control experiments, activity of G6PD alone and G6PD in the presence of HSP-1/2, incubated at 4 °C, was assayed. Activity of the enzyme solution which was incubated in the presence of HSP at 4 °C was found to be almost equal to that of the native enzyme (curve 4). Percent activity of different samples is shown in the form of a bar diagram in Fig. 2D. These results indicate that HSP-1/2 affords protection to substrate proteins against thermal inactivation and lend further to support to the above interpretation that this protein exhibits chaperone-like behavior.

3.2.3. Inhibition of DTT-induced aggregation of insulin by HSP-1/2

Aggregation profiles observed with insulin upon incubation with DTT are shown in Fig. 2E. When DTT is added disulphide bonds in insulin get reduced and the B-chain forms aggregated structures resulting in an increase in the turbidity of the solution, whereas the A-chain remains in solution. Curve 1 in Fig. 2E depicts the aggregation behavior of native insulin in the presence of DTT. Incubation with HSP-1/2 before the addition of DTT resulted in a reduction of the aggregation, which was found to be concentration dependent. Pre-incubation of insulin with HSP-1/2 in 1:0.5 (w/w) ratio (curve 2) reduced the aggregation to 46%. Aggregation was further reduced to 20% and onset of aggregation was delayed when the insulin:HSP-1/2 ratio was decreased to 1:1 (w/w). When the insulin:HSP-1/2 ratio was 1:1.5 (w/w), only 6% aggregation was observed (curve 4). Percent aggregation of insulin in the presence of different concentrations of HSP-1/2 is shown as a bar diagram in Fig. 2F. These results indicate that HSP-1/2 protects insulin from aggregation induced by reducing agents such as DTT.

3.2.4. AFM studies of aggregation-inhibition of LDH by HSP-1/2

Thermal aggregation of target proteins and its inhibition by HSP-1/2 was also investigated by atomic force microscopy and

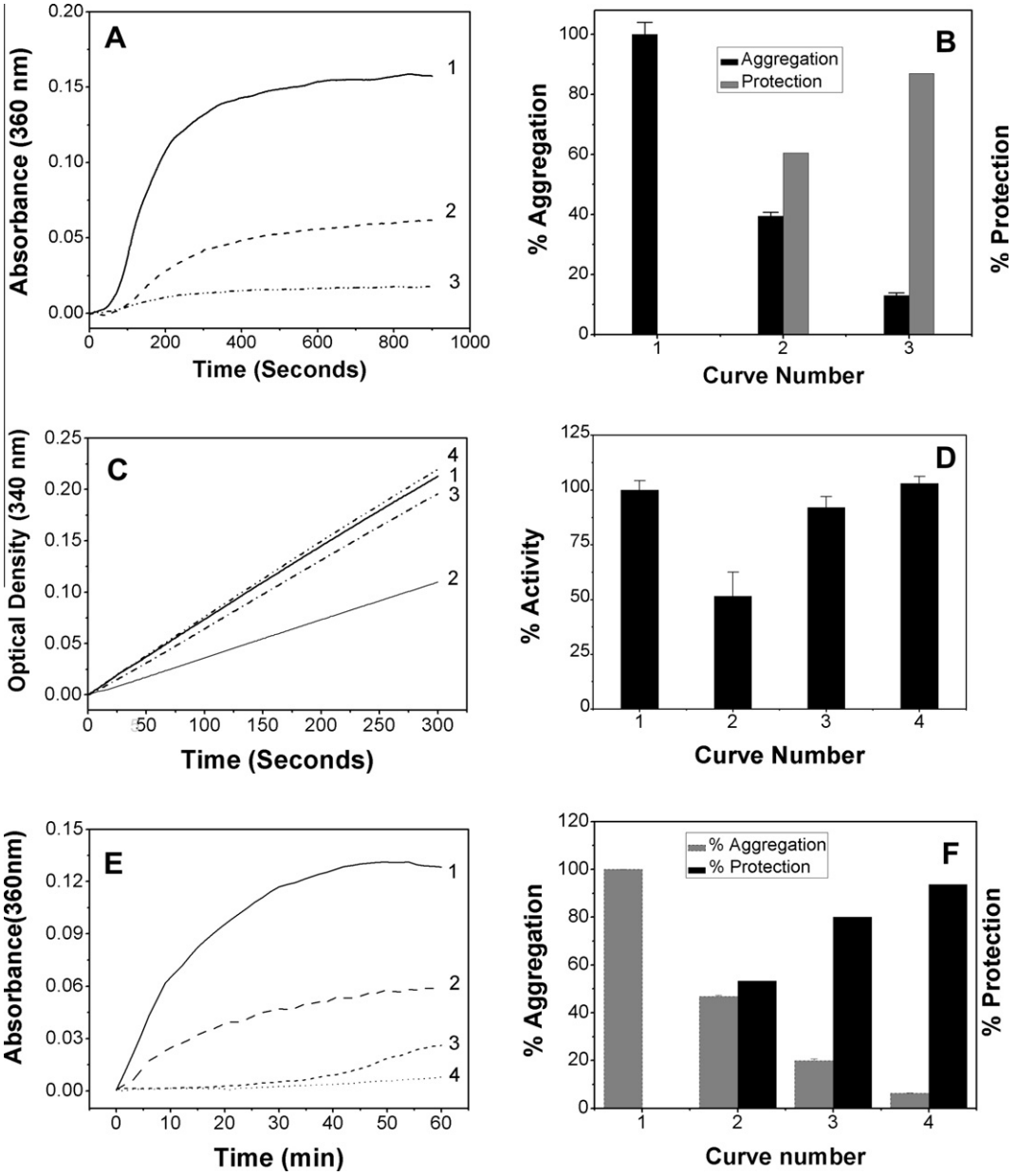

Fig. 2. Chaperone-like activity of HSP-1/2. (A) Prevention of aggregation of ADH (0.05 mg/mL) by HSP-1/2. Aggregation profiles of (1) ADH at 48 °C, (2) ADH + 0.01 mg/mL HSP-1/2 and (3) ADH + 0.025 mg/mL HSP-1/2 are shown. (B) Bar diagram representing percent aggregation (black bars) and protection (gray bars) of ADH by HSP-1/2 at different concentrations. (C) HSP-1/2 assisted reactivation of G6PD. Activity of the enzyme at room temperature under native conditions (1), after incubation at 45 °C (2), upon incubation at 45 °C in the presence of HSP-1/2 (3) and after incubation at 4 °C in the presence of HSP-1/2 (4) are shown. (D) Bar diagram representing the activity of G6PD at 300 s (from panel C). (E) Prevention of DTT induced aggregation of Insulin by HSP-1/2. Aggregation profiles of (1) insulin 0.2 mg/mL, (2) insulin + 0.1 mg/mL HSP-1/2, (3) insulin + 0.2 mg/mL HSP-1/2, and (4) insulin + 0.3 mg/mL HSP-1/2. (F) Bar diagram representing percent aggregation (grey bars) and protection (black bars) of insulin by HSP-1/2 from panel E.

the results obtained with LDH are presented in Fig. 3. AFM images of LDH obtained under native conditions show a uniform size-distribution with small particles (Fig. 3A). Upon incubation at 48 °C the protein yields fairly large aggregates (Fig. 3B). However, when incubated at the same temperature in the presence of HSP-1/2, LDH did not form aggregated structures but remained homogeneous similar to the native protein (Fig. 3C). Distribution density histogram shows a size distribution in the range of 2–25 nm for native LDH (Fig. 3D) and 5–80 nm for heat treated LDH (Fig. 3E). The size distribution shifted toward lower values, viz., 5–35 nm (Fig. 3F) when HSP-1/2 was present along with LDH during heat stress, suggesting that HSP-1/2 prevents the aggregation of the target proteins and functions as a molecular chaperone.

3.3. Inhibition of chaperone-like activity of HSP-1/2 by PrC binding

The effect of PrC binding on the CLA of HSP-1/2 was assessed by turbidimetry and the results obtained are presented in Fig. 4. ADH showed a rapid increase in the turbidity when incubated at 48 °C, which reached a maximum and remained steady thereafter (Fig. 4A, curve 1). Presence of HSP-1/2 (0.01 mg/mL) resulted in a significant reduction in the aggregation (~60% reduction, curve 2). However, preincubation of HSP-1/2 with PrC reversed this in a concentration dependent manner (curves 3–5). ADH alone shows ~50% aggregation at ~150 s (curve 1), whereas in the presence of HSP-1/2 (0.01 mg/mL), even at 900 s the aggregation does not approach 50% of that obtained with ADH alone (curve 2). When

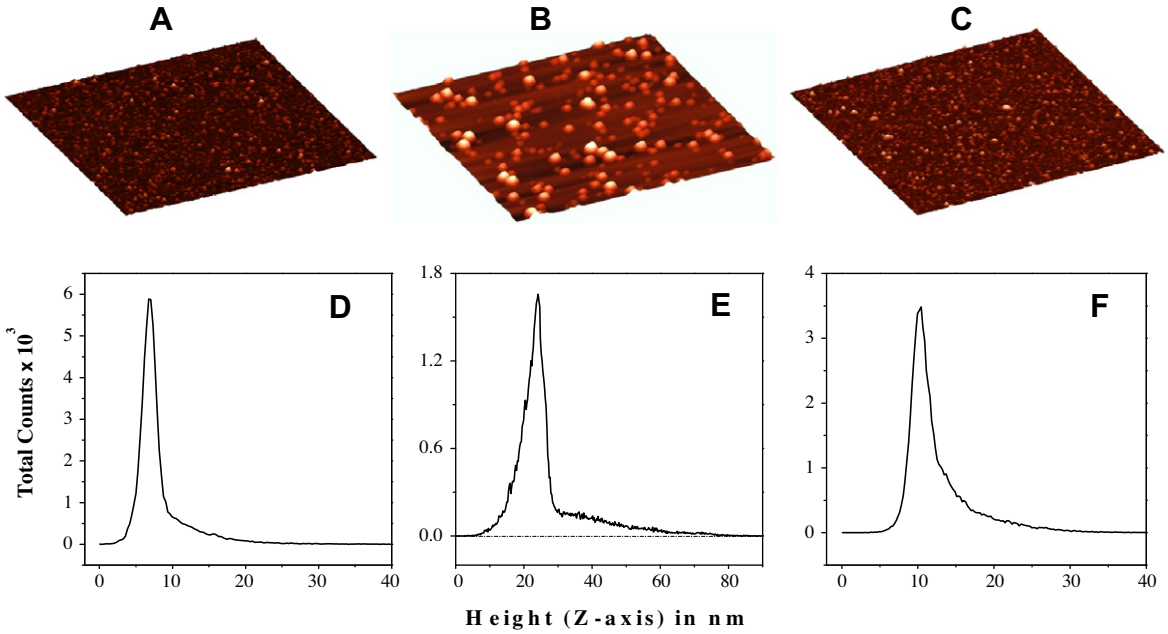

Fig. 3. Prevention of thermal aggregation of LDH by HSP-1/2. AFM images of 0.075 mg/mL LDH in the native state (A), upon heat treatment (B) and upon heat treatment in the presence of 0.075 mg/mL HSP-1/2 (C) are shown. Each image is 5 × 5 μM in size. Distribution density histogram analysis for native LDH (D), upon incubation at 48 °C (E) and upon incubation at 49 °C in the presence of HSP-1/2 (F).

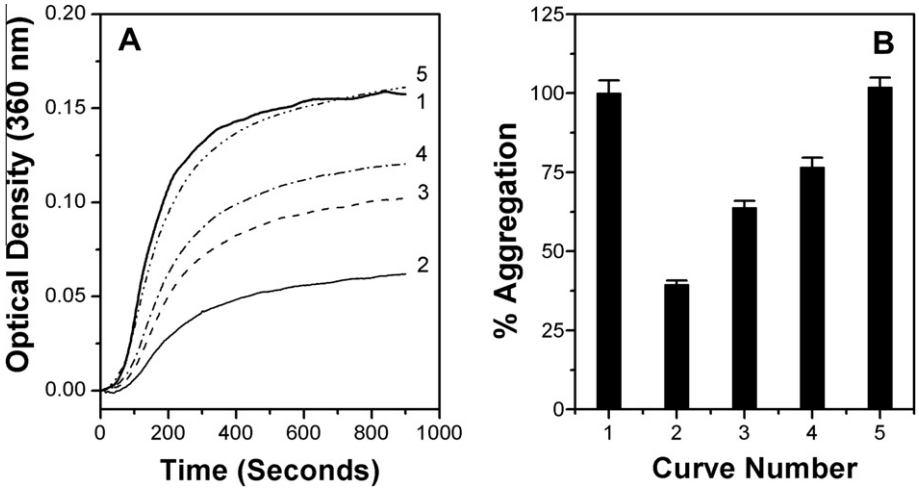

Fig. 4. Effect of phosphorylcholine binding on the chaperone-like activity of HSP-1/2. (A) Aggregation profiles of ADH in the absence and presence of HSP-1/2 and phosphorylcholine. The samples are: (1) ADH at 48 °C, (2) ADH + HSP-1/2, (3) ADH + HSP-1/2 + 0.5 mM PrC, (4) ADH + HSP-1/2 + 1 mM PrC and (5) ADH + HSP-1/2 + 2 mM PrC. ADH and HSP-1/2 concentrations were 0.05 mg/mL and 0.01 mg/mL, respectively in all the samples. (B) Bar diagram representing the percent aggregation of ADH in the various samples.

HSP-1/2 was preincubated with PrC, 50% aggregation was observed at 360, 260 and 165 s when 0.5 mM, 1 mM and 2.0 mM of PrC were used, respectively. A bar diagram representing percent aggregation of ADH observed at 900 s under different conditions is shown in Fig. 4B. These results indicate that PrC binding decreases the CLA of HSP-1/2 in a concentration dependent manner. Similar results were also obtained when LDH was used as the target protein (Fig. S3).

HSP-1/2 was reported to exist in an aggregated structure with a mass of ~90 kDa which in the presence of saturating concentrations of PrC dissociates into smaller aggregates of 25 and 38 kDa [28]. Similar results were reported for the major bovine seminal plasma protein PDC-109 and it was shown that change in the aggregation state of the protein rather than ligand binding *per se* is responsible for the decrease in the CLA of the protein [25]. Therefore, it is likely that for HSP-1/2 also the aggregation state of the protein modulates its chaperone-like activity.

In summary, in this study we have presented several lines of evidence, which together demonstrate that the equine seminal plasma protein, HSP-1/2 exhibits chaperone-like activity. In this respect it is similar to PDC-109, which is the only protein of mammalian seminal plasma that is known to exhibit CLA [25]. This suggests that HSP-1/2 may protect other proteins of equine seminal plasma against misfolding, unfolding or aggregation. CD and computational modeling studies indicate that the structure of HSP-1/2 is largely unordered and it is likely that this structural plasticity helps it to interact with other seminal plasma proteins effectively and protect them under stress conditions. PrC binding

results in a decrease in the CLA of HSP-1/2, indicating that the polydisperse nature of this protein is important for its CLA. These results strongly suggest that seminal plasma proteins that are homologous to HSP-1/2 and PDC-109 may also exhibit chaperone-like activity. Presence of proteins with CLA in the seminal plasma is likely to be of considerable physiological significance, which merits further investigation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.08.120.

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