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Modulation of chaperone-like and membranolytic activities of major horse seminal plasma protein HSP-1/2 by L-carnitine

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The major protein of horse seminal plasma, HSP-1/2, exhibits membranolytic and chaperone-like activities and plays a crucial role in regulating sperm capacitation. L-Carnitine is a small polar molecule present in high concentrations in mammalian seminal plasma. The present results demonstrate that L-carnitine binds to HSP-1/2 and increases its thermal stability, enhances cooperativity of its chemical unfolding and decreases both chaperone-like and membranolytic activities of this protein. The HSP-1/2–L-carnitine complex exhibits anti-oxidative behaviour by inhibiting the production of hydroxyl radicals, suggesting that it can protect other constituents of seminal plasma from damage by hydroxyl radicals. As HSP-1/2 and L-carnitine share the same spatiotemporal location in the horse reproductive tract, this interaction is physiologically significant and may prevent premature interaction of HSP-1/2 with sperm, which in turn regulates the sperm capacitation.

Keywords. Capacitation; membranolytic activity; molecular chaperone; oxidative stress

Abbreviations: ADH, Alcohol dehydrogenase; CLA, Chaperone-like activity; FL, Fluorescein; Gdm.Cl, Guanidinium chloride; HSP-1/2, Horse seminal plasma protein-1/2; PrC, Phosphorylcholine; REES, Red edge excitation shift; ROS, Reactive oxygen species; Trp, Tryptophan; SV plot, Stern–Volmer plot; TBS, 50 mM tris buffer containing 0.15 M NaCl and 5 mM EDTA, pH 7.4

1. Introduction

In mammals, during ejaculation, fibronectin type II (FnII) proteins present in the seminal plasma bind to the sperm surface and play a major role in sperm capacitation. This binding – mediated by the interaction of FnII proteins to choline phospholipids on the sperm plasma membrane – leads to a removal of choline phospholipids and cholesterol, a process referred to as *cholesterol efflux*. This in turn increases fluidity of the plasma membrane and facilitates the acrosome reaction (Austin 1952; Plante *et al.* 2016).

Horse seminal plasma protein, HSP-1/2 is the major FnII protein of equine seminal plasma. It is a non-separable mixture of two proteins: HSP-1, a 121-residue polypeptide with four *O*-glycosylation sites and HSP-2 which is essentially identical to HSP-1, but lacks a 14 amino acid N-terminal segment, which contains two glycosylation sites in the former (Calvete *et al.* 1995). PDC-109, a bovine analog of HSP-1/2, is the best characterized protein of this family. PDC-109 is known to bind several molecules/ligands other than choline phospholipids such as heparin, gelatin, LDL, HDL, apolipoprotein A1 and collagen (Plante *et al.* 2016; Swamy 2004; Sankhala *et al.* 2011). Due to the physiological significance of its interaction with choline phospholipids, several studies have been carried out to investigate the thermodynamics and kinetics of this interaction (Desnoyers and Manjunath 1992; Müller *et al.* 1998; Ramakrishnan *et al.* 2001; Manjunath and Therein 2002; Thomas *et al.* 2003; Swamy 2004). Crystal structure of PDC-109 complexed with phosphorylcholine (PrC) along with fluorescence studies has shown that aromatic amino acids are crucial to this binding and binding of choline phospholipids shield the tryptophan residues from soluble quenchers and Trp-90 gets embedded into the membrane hydrophobic core (Wah *et al.* 2002; Anbazhagan *et al.* 2008). PDC-109 was also shown to extract phospholipids from the membrane resulting in its destabilization whereas presence of cholesterol in the membrane was found to stabilize it from disruption (Swamy *et al.* 2002; Damai *et al.* 2010). Even though HSP-1/2 shares similar binding pattern to choline phospholipids to that of PDC-109, quantitative differences were observed in the extent of membrane immobilization and extraction of lipids from model membranes. Fluorescence studies indicate that the ability of HSP-1/2 to penetrate into the membrane hydrophobic core is weaker as compared

to PDC-109, but exhibits similar membrane destabilization towards model and cell membranes (Greube *et al.* 2004; Kumar *et al.* 2016).

Our group has shown that besides membrane biding property, HSP-1/2 also exhibits chaperone-like activity (CLA) by protecting various target molecules against thermal, chemical and oxidative stress conditions (Sankhala *et al.* 2012; Kumar and Swamy 2016a). Several factors such as pH, ligands and surfactants have been found to modulate the CLA (Kumar and Swamy 2016b, 2017). Membranolytic activity and CLA of HSP-1/2 were found to be pH dependent and inversely correlated to each other, indicating that the dual functionality of HSP-1/2 is regulated by a pH switch (Kumar and Swamy 2016b).

Apart from proteins, seminal plasma also contains several small molecules which are shown to play active role in sperm maturation/protection processes. Carnitine is one such molecule present in significant concentrations (2–100 mM) in mammalian reproductive tract (Soufir *et al.* 1981). Carnitine is a highly polar, soluble and zwitterionic molecule, known for its role (only L-isomer) in acyl chain translocation and regulation of β-oxidation of fatty acid chains in mitochondria. In the reproductive tract, several functions have been attributed to L-carnitine and acylcarnitine, e.g. acting as a buffer system protecting cellular functions, controlling free CoA concentrations, and as a potential initiator for progressive motility of spermatozoa (Juelin and Lewin 1996). The anti-oxidant nature of L-carnitine is also well established (Gulcin 2006) which may have some implications in its presence in systems prone to oxidative stress such as reproductive tract. Further, oral administration of L-carnitine was reported to significantly improve the kinetic and morphological parameters of oligoasthenospermic horses (Stradaioli *et al.* 2004), and in other animals (Kumari and Menon 1988) indicating pharmacological potential of the molecule. Even though several roles have been attributed to L-carnitine, the mechanistic details of how it can play those roles have not been investigated so far. In the present study, we demonstrate that L-carnitine binds to HSP-1/2 and modulates its membranolytic and chaperone-like activities in a concentration-dependent manner.

2. Materials and methods

2.1 *Materials*

L-Carnitine, guanidinium chloride (Gdm.Cl), phosphorylcholine chloride calcium salt (PrC), and heparin-agarose type-I beads were purchased from Sigma (St. Louis, MO, USA). *p*-Aminophenyl phosphorylcholine column was obtained from Pierce Chemical Co. (Oakville, ON, Canada). All other chemicals were obtained from local suppliers and were of the highest purity available.

2.2 *Purification of HSP-1/2*

HSP-1/2 was purified as described earlier from seminal plasma collected from healthy horses by affinity chromatography on heparin-agarose and *p*-aminophenyl phosphorylcholine, followed by reverse phase HPLC (RP-HPLC) (Sankhala *et al.* 2012). The purified HSP-1/2 was extensively dialysed against 50 mM tris buffer, pH 7.4, containing 0.15 M NaCl and 5 mM EDTA (TBS).

2.3 *Circular dichroism spectroscopy*

CD spectroscopic studies were performed using an AVIV 420SF spectropolarimeter (Aviv Biomedical, NJ, USA) fitted with a thermostatted cell holder and a thermostatic waterbath. Samples were taken in a 0.2 cm path length quartz cell and the concentration of HSP-1/2 was ∼0.15 mg/mL and 0.5 mg/mL, respectively, for measurements in the far and near UV regions. Spectra were recorded with native HSP-1/2 alone and in the presence of 10 mM L-carnitine at a scan speed of 20 nm/min. Each spectrum reported is the average of 3 consecutive scans from which buffer scans, recorded under identical conditions, were subtracted.

Thermal unfolding of HSP-1/2 was investigated by monitoring the CD spectral intensity of the protein (0.15 mg/mL) at 223 nm, while the temperature was increased from 25 to 90°C at a scan rate of 1°/min. Effect of L-carnitine binding on the thermal stability of HSP-1/2 was investigated by pre-incubating the protein with L-carnitine (5 or 10 mM) before the measurements.

2.4 *Fluorescence spectroscopy*

Steady-state fluorescence measurements were carried out using a Jobin-Yvon Spex Fluoromax-3 fluorescence spectrometer at room temperature with excitation and emission band pass filters set at 1 and 2 nm, respectively. Protein samples ($A_{280} \leq 0.1$) were excited at 280 nm (for ligand binding studies) or 295 nm (for quenching studies) and the emission spectra were recorded between 310 and 400 nm. Fluorescence quenching experiments were carried out by adding small aliquots of acrylamide from a 5 M stock solution in TBS to HSP-1/2 alone or after pre-incubation with L-carnitine in the same buffer. The quenching data were analysed by Stern–Volmer and modified Stern–Volmer equations. For the red edge excitation shift (REES) experiments excitation wavelength was varied between 280 and 307 nm and emission spectra were recorded between 310 and 400 nm, with a slit width of 1 nm for both excitation and emission monochromators. The results given are the average values of at least two independent experiments (with standard deviations <5% in all cases).

Equilibrium unfolding experiments were performed by mixing a fixed amount of HSP-1/2 (~3 μM) alone or pre-incubated with ligands (20 mM L-carnitine or 20 mM PrC), with increasing amounts of Gdm.Cl and incubated overnight. The samples were excited at 280 nm and emission spectra were recorded in the range of 310 to 400 nm.

2.5 *Aggregation assays*

Aggregation assays to monitor the chaperone-like activity of HSP-1/2 were performed with ADH or aldolase as the target protein (Sankhala *et al.* 2012). Briefly, ADH (0.05 mg) or aldolase (0.1 mg) in TBS was mixed with HSP-1/2 alone or HSP-1/2 pre-incubated with L-carnitine (0.5–10 mM) for 5 min before carrying out the assay at 48°C. Aggregation of the target protein alone was taken as 100% and aggregation of other samples was normalized with respect to it. Average values from at least two independent experiments have been reported.

2.6 *Fluorescein assay*

Hydroxyl radical detection assay was performed using fluorescein (FL) essentially as described earlier (Kumar and Swamy 2016a). Fluorescence decay profile of FL was monitored at 515 nm in an ISS PC1 fluorescence spectrometer (Champaign, IL) by exciting at 493 nm. The initial fluorescence intensities of fluorescein (after the addition of Co^{2+}) were normalized. The percent inhibition of hydroxyl radical was calculated as described in (Kumar and Swamy 2016a). Average values from at least two independent experiments are reported.

2.7 *Erythrocyte lysis assay*

Erythrocyte lysis assay was performed as described earlier (Kumar and Swamy 2016b). A 100 μL aliquot from a 4% human erythrocyte suspension in TBS was mixed with 50 μg of HSP-1/2 alone or after pre-incubation with different concentrations of L-carnitine. The samples were then incubated for 1 h, centrifuged at 5000 rpm for 5 min and absorbance of the supernatant was measured at 415 nm.

2.8 *Confocal microscopy*

Confocal microscopic images of human erythrocytes in the presence of HSP-1/2, L-carnitine and their combination were obtained using a Leica TCS SP2 confocal microscope (Heidelberg, Germany) as described earlier (Kumar and Swamy 2016a). To investigate the effect of ligand binding, HSP-1/2 was pre-incubated with 10 mM L-carnitine for 10 min before its addition to the erythrocyte suspension. Imaging was performed after incubating the samples for 60 min.

3. Results and discussion

3.1 *CD spectroscopic studies of HSP-1/2 in presence of L-carnitine*

Effect of L-carnitine on the secondary and tertiary structure of HSP-1/2 was investigated using CD spectroscopy. Far-UV CD spectra of HSP-1/2 alone and in the presence of 10 mM L-carnitine are shown in figure 1A. The spectrum of the protein alone (black line) contains a broad positive asymmetric band with maximum at 223 nm, which is essentially unchanged in the presence of L-carnitine (red line). The near-UV CD spectrum of HSP-1/2 contains two overlapping positive bands with maxima at ~282 and ~288 nm (figure 1B) and presence of L-carnitine resulted in an increase in the signal intensity with no major changes in the shape of the spectrum (Sankhala *et al.* 2012). Overall these results indicate that L-carnitine binding does not induce any significant changes in the secondary and tertiary structure of HSP-1/2.

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 (figure 1C, black line). Midpoint of the unfolding transition of native HSP-1/2, observed at ~46°C, is consistent with previous results (Sankhala *et al.* 2012). In the presence of 5 mM and 10 mM L-carnitine the transition midpoint shifted to ~53°C (red line), and ~57°C (blue line), respectively. A further shift to 59 °C was observed when the concentration of L-carnitine was increased to 20 mM (data not shown). These results clearly show that binding of L-carnitine stabilizes the structure of HSP-1/2 in a concentration dependent manner, which is similar to the observations made regarding the binding of PrC to HSP-1/2 (Sankhala *et al.* 2012).

3.2 *Fluorescence studies on interaction of HSP-1/2 with L-carnitine*

Fluorescence spectra of HSP-1/2 alone and in the presence of phosphorylcholine (PrC) and L-carnitine are shown in figure 2A and the changes observed in the emission characteristics are presented in table 1. The results presented in figure 2A and table 1 indicate that while the blue shift in the emission maximum induced by both PrC and L-carnitine are comparable, the ligand-induced increase in emission intensity of the protein intrinsic fluorescence is more for PrC

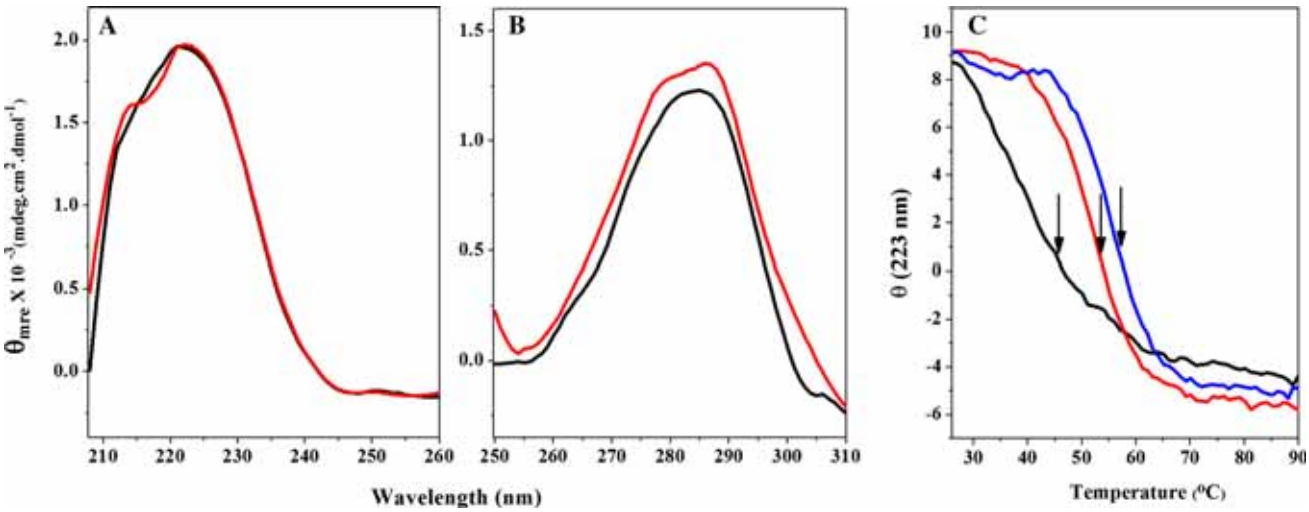

Figure 1. Circular dichroism studies on L-carnitine interaction with HSP-1/2. (**A**) Far-UV and (**B**) Near-UV CD spectra of HSP-1/2 alone (*black line*) and in the presence of 10 mM L-carnitine (*red line*). (**C**) CD thermal scans of HSP-1/2 alone (*black line*) and in the presence of L-carnitine (*red line*, 5 mM; *blue line*, 10 mM).

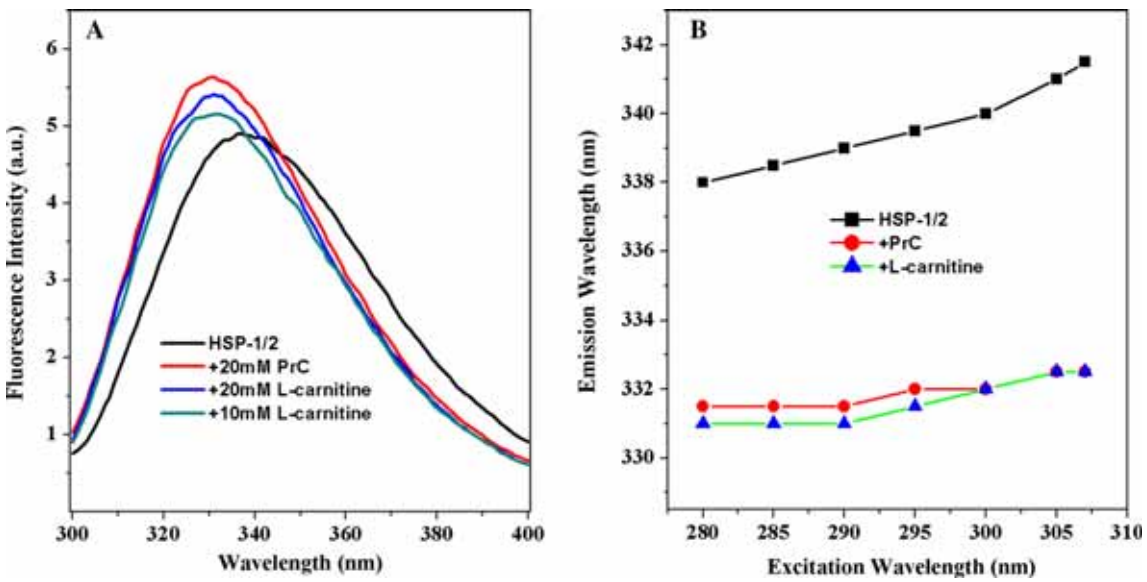

Figure 2. Fluorescence spectroscopic studies on HSP-1/2 interaction with PrC and L-carnitine. (**A**) Intrinsic fluorescence spectra of HSP-1/2 alone and in presence of PrC and L-carnitine. (**B**) Effect of change in excitation wavelength on the emission maximum of HSP-1/2 alone (*squares*), in presence of 20 mM PrC (*circles*) and in presence of 20 mM carnitine (*triangles*).

than L-carnitine. HSP-1/2 shows a red edge excitation shift (REES) of 3.5 nm when the excitation wavelength is changed from 280 to 305 nm indicating that water molecules near Trp residues are under motional restriction (Kumar *et al.* 2016). Addition of PrC and L-carnitine reduced the REES to 1 and 1.5 nm, respectively (figure 2B), indicating that binding both PrC and L-carnitine results in a displacement of water molecules and suggests that both these ligands may occupy the same binding pocket. In addition, there was no change in the blue shift or emission intensity when L-carnitine was added to HSP-1/2 preincubated with PrC. Similarly, when PrC was added to HSP-1/2–L-carnitine complex, no blue shift was observed with only a marginal increase in the emission intensity (data not shown).

Table 1. Parameters obtained from steady-state fluorescence quenching of HSP-1/2 by acrylamide in the absence and in presence of different ligands

Sample description	λ_{max} (nm)	Fluorescence change (%)	Quenching (%)	K_{SV1} (M^{-1})	K_{SV2} (M^{-1})	F_a (%)	K_a (M^{-1})
Native HSP-1/2	338	–	88.5	8.48	–	91.7	10.1
+ 20 mM PrC	331.5	15.1	55.4	2.15	3.9	68.1	9.34
+ 20 mM L-Carnitine	331	10.5	49.5	2.11	–	62.5	6.74

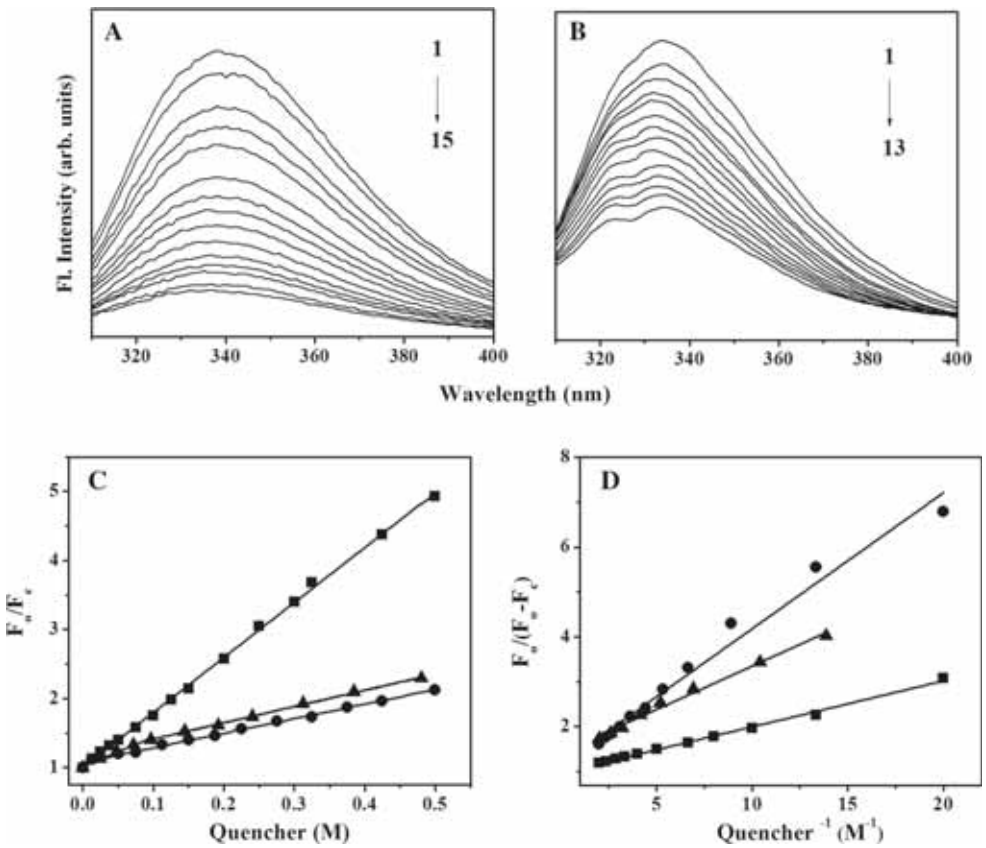

Figure 3. Fluorescence quenching studies. Fluorescence spectra of (**A**) native HSP-1/2 and (**B**) in presence of 20 mM L-carnitine in the absence and in the presence of various concentrations of acrylamide are shown. Stern–Volmer (**C**) and modified Stern–Volmer (**D**) plots of quenching data under different conditions: (*squares*) HSP-1/2 alone, (*triangles*) in the presence of 20 mM PrC and (*circles*) in presence of 20 mM L-carnitine.

These results strongly suggest that both L-carnitine and PrC may occupy the same binding pocket.

In order to investigate this further, we performed fluorescence quenching studies. Figure 3A and B show fluorescence spectra corresponding to quenching of HSP-1/2 by acrylamide in the native state and in the presence of L-carnitine, respectively. In each case, the spectrum exhibiting the highest intensity corresponds to HSP-1/2 in the absence of acrylamide, and the spectrum with the lowest intensity corresponds to that recorded in the presence of 0.5 M acrylamide. The remaining spectra correspond to those recorded in the presence of different concentrations of the quencher and in each case as the quencher concentration is increased, the fluorescence intensity was found to decrease. Consistent with previous reports, significant quenching ($\sim$88.5%) was observed for native HSP-1/2 in the presence of 0.5 M

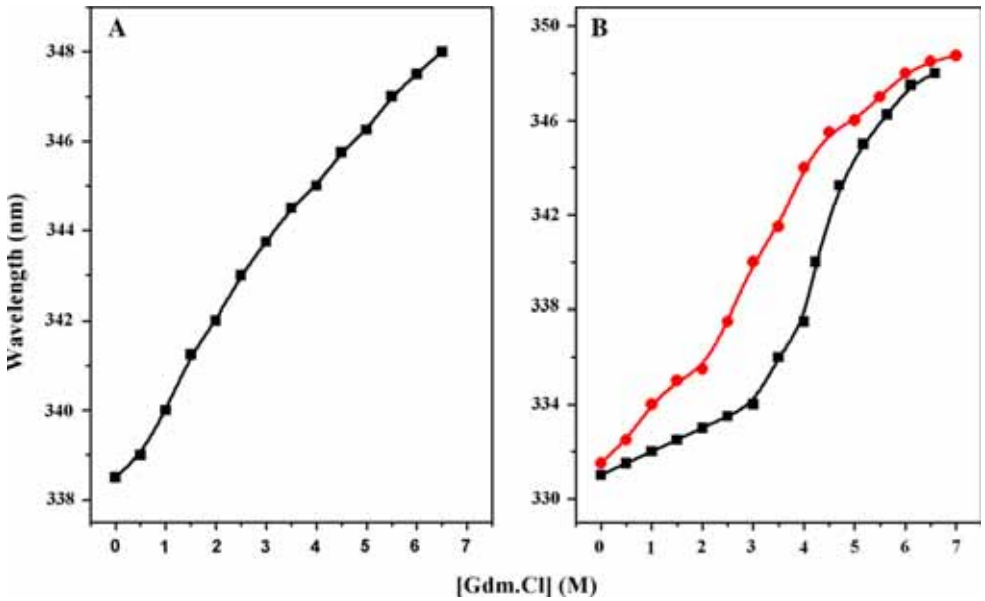

Figure 4. Effect of L-carnitine on the chemical unfolding of HSP-1/2. Change in emission maximum of HSP-1/2 under different conditions is plotted as a function of denaturant (Gdm.Cl) concentration. (**A**) Native HSP-1/2 and (**B**) HSP-1/2 in presence of 20 mM L-carnitine (*red symbols*) and 20 mM PrC (*black symbols*).

acrylamide, whereas in the presence of 20 mM L-carnitine the quenching decreased to 49.5%. These observations parallel the results obtained for quenching of HSP-1/2 in presence of PrC (Kumar *et al.* 2016).

The data obtained for acrylamide quenching of HSP-1/2 alone and upon pre-incubation with carnitine were analysed by the Stern–Volmer equation (equation 1) and modified Stern–Volmer equation (equation 2) given below (Kumar *et al.* 2016):

$$F_o/F_c = 1 + K_{SV}[Q] \quad (1)$$

$$F_o/(F_o - F_c) = f_a^{-1} + 1/(K_a f_a[Q]) \quad (2)$$

where F_o and F_c are fluorescence intensities of the HSP-1/2 recorded in the absence and presence of quencher, respectively. [Q] is acrylamide concentration, K_{SV} is the Stern–Volmer constant, f_a is the fraction of total fluorophores accessible to the quencher and K_a is the corresponding Stern–Volmer constant for the accessible fraction of fluorophores.

Stern–Volmer (SV) and modified Stern–Volmer plots of HSP-1/2 alone and in presence of L-carnitine are shown in figure 3C and D, respectively. For comparison, plots obtained for HSP-1/2 in presence of PrC with acrylamide from previously reported data (Kumar *et al.* 2016a) are also presented. While the SV plots show a biphasic quenching pattern in the presence of PrC, the plots obtained in the presence of L-carnitine are monophasic indicating that while PrC induces heterogeneity of Trp residues in HSP-1/2, L-carnitine does not. The fraction of fluorophores (f_a) obtained from the intercept of the modified SV plots were comparable for PrC (~68%) and L-carnitine (62.5%). These results are summarized in table 1. All these results suggest strong similarity in the interaction of both PrC and L-carnitine with HSP-1/2.

3.3 *Effect of L-carnitine binding on the chemical unfolding of HSP-1/2*

Chemical unfolding of HSP-1/2 by Gdm.Cl was studied by fluorescence spectroscopy as described earlier (Kumar *et al.* 2016) and unfolding plots depicting λ_{max} of HSP-1/2 as a function of Gdm.Cl concentration are given in figure 4. Consistent with previous results (Kumar *et al.* 2016), unfolding of native HSP-1/2 exhibited low cooperativity, and pre-incubation with PrC gave cooperative unfolding curves with the transition centered at 4.3 M Gdm.Cl. When unfolding experiments were carried out after pre-incubating HSP-1/2 with 20 mM L-carnitine, similar cooperative unfolding transition centered at 3.1 M was observed with the emission λ_{max} shifting from 331 to 348.5 nm. This indicates that similar to PrC, L-carnitine induces changes in the structure/aggregation state of HSP-1/2 which in turn induces cooperativity in the chemical unfolding transition. Our earlier studies showed that cooperativity of unfolding of

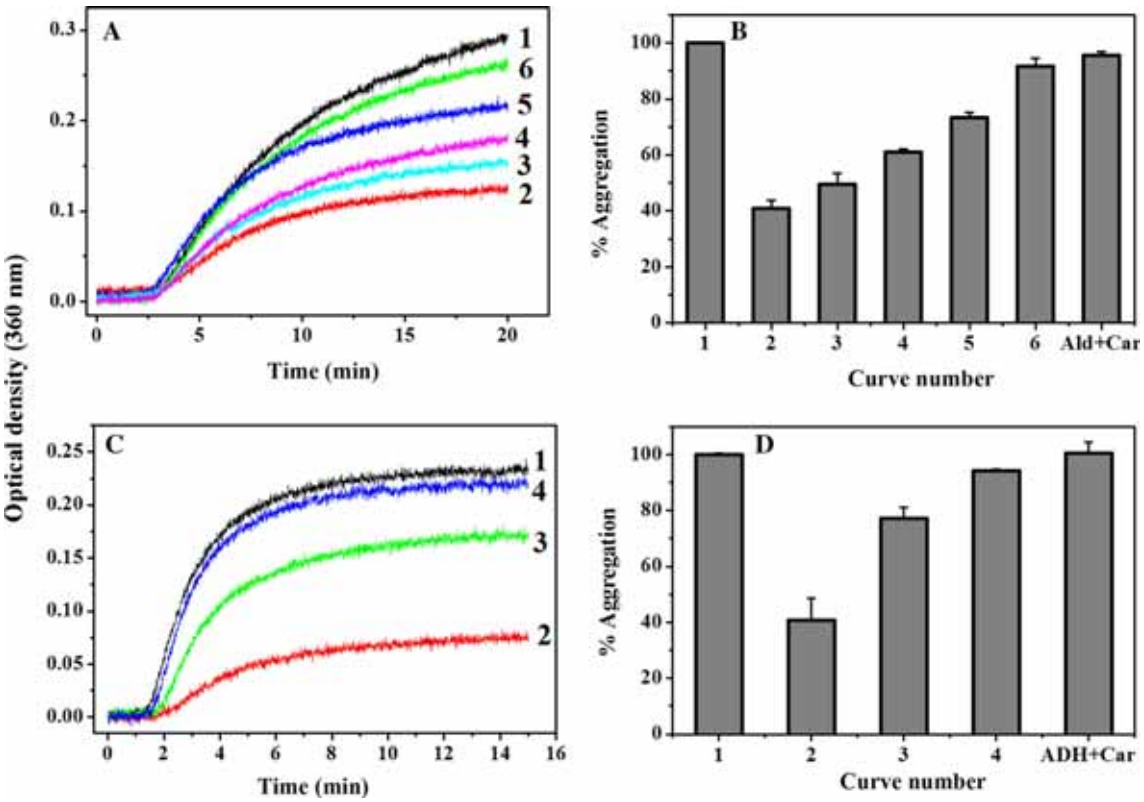

Figure 5. Effect of L-carnitine binding on the CLA of HSP-1/2. (**A**) Aggregation profiles of aldolase (0.1 mg/mL) in the absence and presence of HSP-1/2 (0.02 mg/mL) and L-carnitine at 48 °C. The samples are: (1) aldolase alone, (2) with HSP-1/2, (3) with HSP-1/2 + 0.5 mM L-carnitine, (4) with HSP-1/2 + 1 mM L-carnitine, (5) with HSP-1/2 + 2 mM L-carnitine, (6) with HSP-1/2 + 5 mM L-carnitine. (**B**) *Bar graph* representing the aggregation of aldolase in the various samples. (**C**) Aggregation profiles of ADH (0.05 mg/mL) in the absence and presence of HSP-1/2 and L-carnitine at 48 °C. The samples are: (1) ADH alone, (2) with HSP-1/2, (3) with HSP-1/2 + 5 mM L-carnitine, (4) with HSP-1/2 + 10 mM L-carnitine. (**D**) *Bar graph* representing the aggregation of ADH in the various samples. HSP-1/2 concentration was 0.02 mg/mL in all the samples where it was used.

HSP-1/2 depends on its polydisperse nature and reduction of the polydispersity via ligand binding induces the cooperativity of the unfolding (Kumar *et al.* 2016). The present results suggest that a similar change in the polydispersity, resulting from L-carnitine binding may be responsible for the increased cooperativity of the Gdm.Cl-induced unfolding of HSP-1/2.

3.4 *Effect of L-carnitine binding on CLA of HSP-1/2*

Effect of L-carnitine binding on the CLA of HSP-1/2 was assessed by turbidimetric assays using aldolase and ADH as target proteins and the results obtained are presented in figure 5. Aldolase showed a rapid increase in the turbidity when incubated at 48°C, which reached a maximum and remained steady thereafter (figure 5A, curve 1). Presence of HSP-1/2 (0.02 mg/mL) resulted in ∼60% reduction in the aggregation (curve 2). Pre-incubation of HSP-1/2 with L-carnitine decreased this protective action and reversed it in a concentration dependent manner. When HSP-1/2 was pre-incubated with 0.5 mM L-carnitine the aggregation of aldolase increased to 50%. In the presence of 1 mM and 2 mM L-carnitine aggregation increased to 61% and 73%, respectively. When the L-carnitine concentration was increased to 5 mM, ∼92% aggregation was observed indicating further decrease in protective activity of HSP-1/2. L-Carnitine alone did not affect the aggregation of aldolase as shown in the bar diagram. Similar results were obtained when ADH was used as a target enzyme. This inhibition of CLA of HSP-1/2 by L-carnitine is very similar to that observed for reduction of CLA of HSP-1/2 by PrC. This was attributed to ligand binding-induced decrease in polydispersity of the protein (Sankhala *et al.* 2012; Gasset *et al.* 1997). As similar results were obtained in this study with L-carnitine, a similar mechanism is likely to be operative in this case as well.

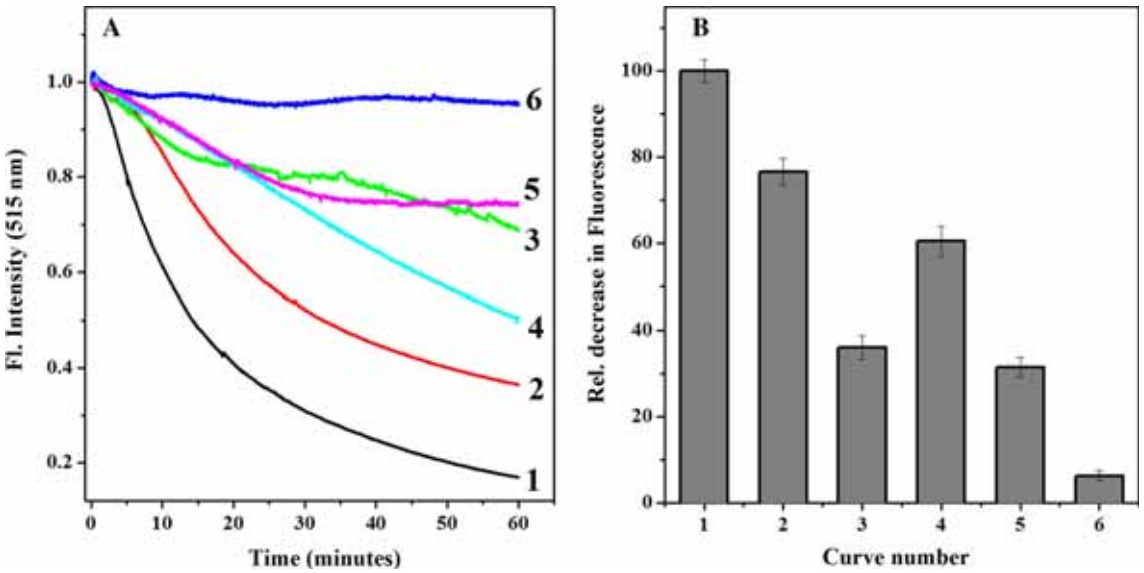

Figure 6. Fluorescein assay for prevention of hydroxyl radical. (**A**) Loss of fluorescence of fluorescein induced by Co^{2+}/H_2O_2 system (1) and effect of various additives: 5 mM L-carnitine (2), 10 mM L-carnitine (3), HSP-1/2 (4), HSP-1/2 + 5 mM L-carnitine (5), and HSP-1/2 + 10 mM L-carnitine (6). HSP-1/2 concentration was 25 μg/mL wherever it was used. (**B**) *Bar graph* representing relative decrease in the fluorescence intensity.

3.5 *Effect of L-carnitine binding on hydroxyl radical prevention by HSP-1/2*

Recent studies have shown that HSP-1/2 exhibits chaperone-like activity by protecting various enzymes and proteins against reactive oxygen species (Kumar and Swamy 2016a). While investigating the effect of HSP-1/2–L-carnitine complex on the hydroxyl radical generating system using FL as a probe, we found that L-carnitine itself was able to prevent the hydroxyl radical production in a concentration dependent manner as indicated by a decrease in the loss of fluorescence of FL (figure 6). In the presence of 5 mM and 10 mM L-carnitine the loss of fluorescence observed was ∼77% and ∼38%, respectively. This result is consistent with the anti-oxidative property of L-carnitine (Gulcin 2006). When L-carnitine and HSP-1/2 were employed together, an additive effect was seen in the protection against loss of fluorescence of FL. At 25 μg/mL concentration of HSP-1/2 the loss of fluorescence was ∼41%, whereas in the presence of HSP-1/2 and 5 mM L-carnitine, the observed loss of fluorescence was ∼30%. When the assay was performed in the presence of HSP-1/2 and 10 mM L-carnitine, the loss of fluorescence further decreased to ∼6%. These results indicate that HSP-1/2–L-carnitine complex acts as an effective hydroxyl radical inhibitory system. These results are of significant interest as both HSP-1/2 and L-carnitine are present in the reproductive system, which frequently experiences oxidative stress. The presence of HSP-1/2–L-carnitine complex can counter the radical induced oxidative stress and protect spermatozoa and other proteins efficiently. It is also of commercial importance as sperm stored in semen extenders shows only ∼30% recovery with major cause of loss attributed to oxidative stress (Baumber *et al.* 2001). Hence addition of HSP-1/2 and L-carnitine may be used to minimize these deleterious effects.

3.6 *Effect of L-carnitine binding on membrane destabilization by HSP-1/2*

Effect of L-carnitine binding on the membranolytic activity of HSP-1/2 was assessed by erythrocyte lysis assay using erythrocytes as model cell membranes and the results obtained are shown in figure 7A. Our previous work has shown that HSP-1/2 destabilizes (lyses) erythrocyte membrane by specifically binding to choline phospholipids present on it, and that blocking the choline binding site by adding PrC greatly reduces the membrane lysis. (Kumar and Swamy 2016b). Preincubation of HSP-1/2 with L-carnitine also decreased the erythrocyte lysis in a concentration dependent manner. Interestingly, L-carnitine was more effective than PrC in decreasing the erythrocyte lysis by HSP-1/2, i.e. higher concentrations of PrC (20 mM, 8 fold) was required to impart the same effect of L-carnitine at low concentrations (2.5 mM).

The above results were further confirmed by confocal microscopic studies. Human erythrocytes without any additive were found to be ∼6–8 μm in size with intact cell membrane (figure 7B). In agreement with previous results

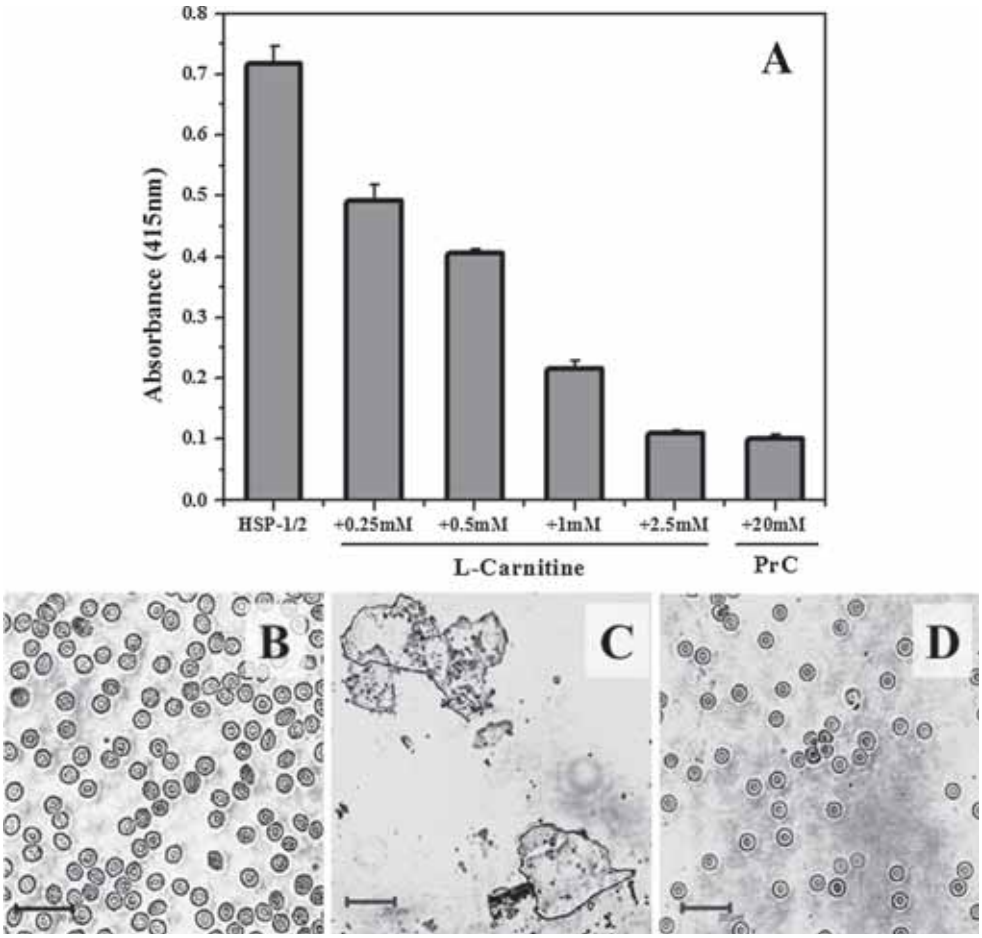

Figure 7. Erythrocyte lysis by HSP-1/2. (**A**) Effect of pre-incubation with PrC and L-carnitine on erythrocyte lysis by HSP-1/2. The concentrations of L-carnitine and PrC used are indicated. (**B**)–(**D**) Confocal images of human erythrocytes in: (**B**) buffer, (**C**) after incubation with 100 µg/mL HSP-1/2 for 1 h, and (**D**) after incubation for 1 h with HSP-1/2 pre-treated with 10 mM L-carnitine.

(Kumar and Swamy 2016b), incubation with 100 µg/mL HSP-1/2 for 1 h resulted in extensive cell lysis and only fragmented membranes could be seen in the confocal images (figure 7C). Pre-incubation of HSP-1/2 with 10 mM L-carnitine for 15 min resulted in a complete inhibition of the membranolytic activity of HSP-1/2 and normal looking and intact erythrocytes were observed (figure 7D).

4. Conclusions

In summary, the present work demonstrates that L-carnitine, a metabolite present in seminal plasma at high concentrations, interacts with HSP-1/2 and modulates its properties. Binding of L-carnitine was found to increase the thermal stability of HSP-1/2, shield its tryptophan residues from quenchers, enhance cooperativity of chemical unfolding and decrease both chaperone-like and membranolytic activities of this protein. The HSP-1/2–L-carnitine complex exhibits anti-oxidative behaviour by inhibiting the production of hydroxyl radicals, suggesting that it can function as an active protective system against hydroxyl radicals in the reproductive tract. It is well known that spermatozoa in the proximal parts of the epididymal lumen contain undetectable amounts of L-carnitine, whereas its concentration in the spermatozoa increases significantly during their passage through the epididymis (Juelin *et al.* 1987; Juelin and Lewin 1996). It may be noted that HSP-1/2 is also released into the reproductive tract in the epididymis region in high concentrations and its immediate interaction with spermatozoa may cause premature capacitation, which is physiologically unfavorable (Ekhlasi-Hundrieser *et al.* 2004). Thus, the HSP-1/2–L-carnitine interaction may prevent premature capacitation of the spermatozoa. These results further our

understanding of interacting partners of seminal FnII proteins and shed light on their regulatory roles in mammalian reproduction.

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