Biochemical and Biophysical Characterization of Chaperone-like Activity and Lipid Binding to the Major Protein of Horse Seminal Plasma, HSP-1/2

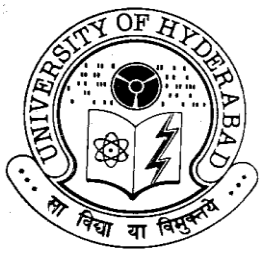
A thesis

Submitted for the degree of

DOCTOR OF PHILOSOPHY

By

C. Sudheer Kumar



School of Chemistry

University of Hyderabad

Hyderabad – 500 046

INDIA

December 2015

I humbly dedicate this thesis to.....

My Parents, Family & Friends

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STATEMENT

I hereby declare that the matter embodied in this thesis is the result of investigations carried out by me in the School of Chemistry, University of Hyderabad, Hyderabad, under the supervision of **Prof. Musti J. Swamy**.

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made whenever the work described is based on the finding of other investigators. Any omission which might have occurred by oversight or error is regretted.

Hyderabad

December 2015 **C. Sudheer Kumar**

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CERTIFICATE

Certified that the work embodied in this thesis entitled **"Biochemical and Biophysical Characterization of Chaperone-like Activity and Lipid Binding to the Major Protein of Horse Seminal Plasma, HSP-1/2"** has been carried out by **Mr. C. Sudheer Kumar** under my supervision and the same has not been submitted elsewhere for any degree.

Hyderabad

December 2015

Prof. Musti J. Swamy

(Thesis Supervisor)

Dean

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DECLARATION

I, **C. Sudheer Kumar** hereby declare that the thesis entitled **"Biochemical and Biophysical Characterization of Chaperone-like Activity and Lipid Binding to the Major Protein of Horse Seminal Plasma, HSP-1/2"** submitted by me under the supervision of Prof. **Musti J. Swamy** is a bonafide research work which is free from plagiarism. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma. I hereby agree that my thesis can deposited in Shodganga/INFLIBNET.

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Sudheer

ABBREVATIONS

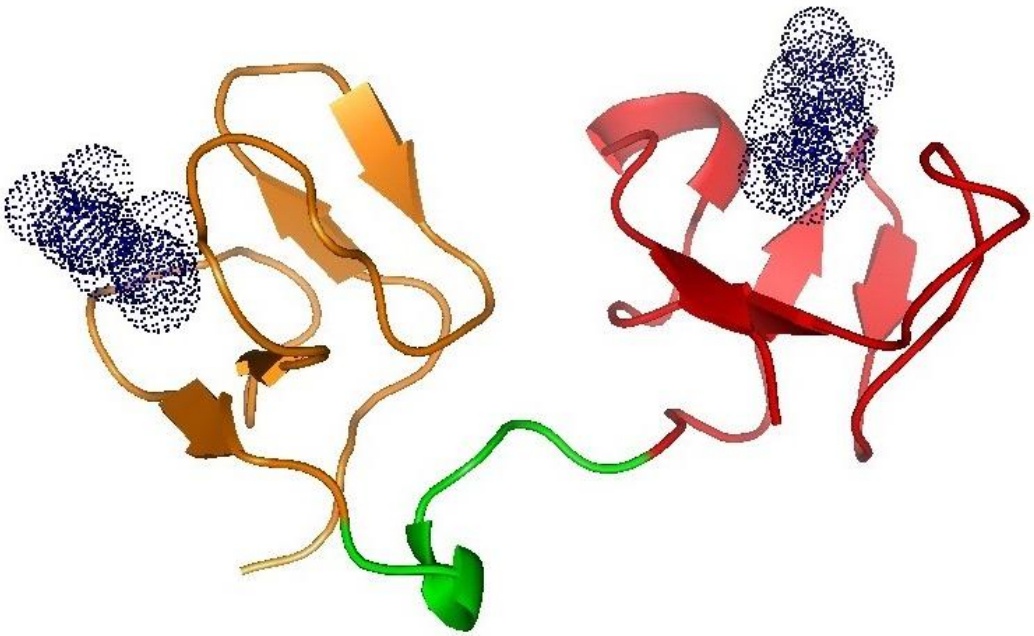
AD	Alzheimer's disease
ADH	Alcohol dehydrogenase
AFM	Atomic force microscopy
ANS	8-anilino-1-naphthlene-sulphonic acid
ATP	Adenosine triphosphate
AR	Acrosome reaction
BSP	Bovine seminal plasma
ΔC_p	Change in excess heat capacity
CA	Carbonic anhydrase
cAMP	cyclic adenosine monophosphate
CD	Circular dichroism
CDNB	1-chloro-2,4-dinitrobenzene
CF	Cystic fibrosis
Chol	Cholesterol
CLA	Chaperone-like activity
CRAC	Cholesterol recognition amino acid consensus
Da	Dalton
DNA	Deoxyribonucleic acid

DMPC	1,2-dimyristoyl-*sn*-glycero-3-phosphocholine
DSC	Differential scanning calorimetry
DTT	Dihiothreitol
EDTA	Ethylenediamine tetra acetic acid
ESR	Electron spin resonance
Fn	Fibronectin
ΔG	Change in free energy
GAG	Glycosaminoglycans
Gdm.Cl	Guanidinium chloride
Gdm.SCN	Guanidinium thiocyanate
GlcNAc	*N*-acetylglucosamine
G6PD	Glucose-6-phosphate dehydrogenase
GST	Glutathione-S-transferase
ΔH	Change in enthalpy
HCl	Hydrochloric acid
His	Histidine
HPLC	High performance liquid chromatography
Hsp	Heat shock protein
ITC	Isothermal titration calorimetry

K_a	Association constant
LDH	Lactate dehydrogenase
βME	β-mercaptoethanol
MRE	Mean residue ellipticity
MRW	Mean residue weight
NaCl	Sodium chloride
NADP	Nicotinamide adenine dinucleotide phosphate
NMR	Nuclear magnetic resonance
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PD	Parkinson's disease
PDB	Protein data bank
pI	Isoelectric point
PrC	Phosphorylcholine
Pro	Proline
RBC	Red blood cells
REES	Red-edge excitation shift

ROS	Reactive oxygen speciess
RNA	Ribonucleic acid
ΔS	Change in entropy
SDS	Sodium dodecyl sulfate
SPR	Surface plasmon resonance
SUV	Small unilamellar vesicles
TBS I	Tris buffer containing 50mM Tris, 0.15M NaCl, 5mM EDTA, pH=7.4
TFA	Trifluoroacetic acid
T_m	Transition mid temperature
ThT	Thioflavin T
Trp	Tryptophan
Ts	Transverse section
Tyr	Tyrosine
ZP	Zona pellucida

Introduction



3D Model of PDC-109 complexed with PrC (adopted from Plante et al., 2015)

Introduction

Reproduction is the biological process by which organisms produce/generate new off springs of the same kind. Reproduction can be broadly classified into two categories - sexual and asexual. Asexual reproduction is carried out by the individual organisms alone, without involving another individual. In case of sexual reproduction, male and female gametes, commonly called sperm and egg respectively, interact and fuse with one another resulting in the formation of new individual organism.

1.1. Male and female gametes

1.1.1. Oocyte (egg)

Oocyte or egg or ovum is the female germ cell that is involved in the reproduction. Ova are produced by a process known as 'oogenesis', which occurs in the ovary. In mammals, the germinal epithelium of the ovary develops into the ovarian follicles which are the functional units of ovary. The development of mature ovum is a highly complex process and involves the development of 'oogonium' and 'primary oocyte' which are diploid in nature and to finally haploid 'secondary oocyte' whose development will be halted at metaphase-II until fertilization. After fertilization the secondary oocyte will undergo maturation process and transforms into mature ovum.

1.1.2. Sperm

The term 'sperm' is derived from the greek word '*sperma*' meaning 'seed' and is the male germ cell which participate in the reproduction. Sperm cells were first observed by Anton Von Leeuwonhoek in 1677. Spermatozoa are produced by a process known as 'spermatogenesis', which occurs in the testis. The sperm cells are produced from the initial, immature spermatogonia from the male primordial germ cells present in the basal epithelium of testis. These spermatogonia through mitotic division form primary spermatocytes. The primary spermatocytes divide meiotically into two secondary spermatocytes. Each secondary spermatocyte undergoes second

meiotic division to produce two spermatids, which develop into mature spermatozoa. Thus each spermatogonium will produce four spermatozoa. The maturation of spermatids to spermatozoa is termed as '*spermiogenesis*'. This process involves changes in the shape and size of the sperm cell and involves nuclear condensation, acrosome formation and development of flagellum from axonemal structure near centrioles [Clermont et al., 1993, de Krester & Kerr, 1994].

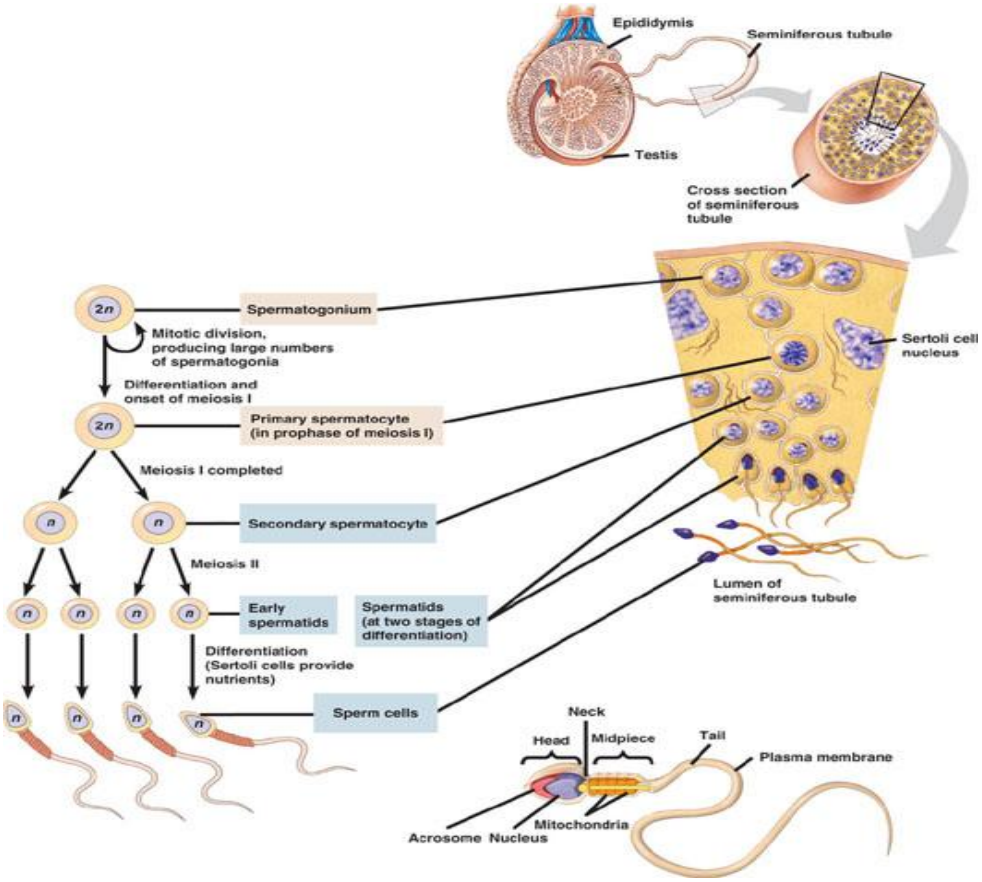


Fig. 1.1. Spermatogenesis. (Taken from porpax.bio.miami.edu/~cm).

1.1.3. Structure of mammalian sperm

Mammalian spermatozoa which perform the function of carrying genetic material exhibit a great variety of shape, size and form among various animal groups. But the structure of the sperm is highly conserved and uniform throughout the mammalian species. Sperm is the only cell that has the ability to swim in mammalian tissue system. A mature sperm contains four parts viz. head, neck, middle piece and tail [Phillips, 1975].

Head is conical in shape and consists of acrosome and nucleus. Acrosome is found on the anterior tip of the sperm head and is formed from the golgi complex during spermatogenesis. This alone is bound by a membrane and contains various hydrolytic enzymes such as phosphatases, lysozymes etc. which help in the lysis of egg membrane and facilitates the penetration of sperm into the egg. Sperm nucleus occupies most of the volume of the sperm head and is covered by a nuclear membrane. Nucleus is devoid of any fluid content and nucleolus, but contains DNA and some basic proteins.

Neck is a short piece that separates head and middle piece. Neck mainly consists of two centrioles called proximal centriole and distal centriole. Both are present in the posterior depression of the neck. They enter into the egg along with the nucleus and are essential for the primary divisions of zygote by forming the first mitotic spindle fiber.

Middle piece consists of outer ring of the coarse fibers and the mitochondrial sheath around it. The mitochondria arranged end to end constitute a helix around the two longitudinal fibers spread along the tail, which is the main source of energy for the sperm motility. These longitudinal fibers are called beta fibers, which are surrounded by nine pairs of fibers called alpha fibers. Together they form a circular 9+9+2 cross sectional pattern [Fawcett, 1975; Phillips, 1975].

Introduction

Tail is usually the longest part of the sperm cell. It mainly constitute of two parts, principle piece and end piece. The tail is made up of axial filaments with 9+2 arrangement. These fibrils are surrounded by fibrous sheath which sometimes appear as helical coils. The distinct arrangement of these fibers throughout the tail in three and four perpendicular to each other helps more powerful stroke of tail in one direction and hence in the sperm motility.

1.1.4. Sperm capacitation

Unlike the sperm of may lower species, even though ejaculated mammalian sperm are motile and appear mature morphologically, they do not possess the ability to fertilize the egg. Ejaculated sperm has to spend a definite amount of time in the female reproductive track to attain fertilizing ability. This time dependent changes in sperm that enable sperm to attain capacity to fertilize an egg is defined as 'capacitation' [Chang, 1951 & Austin, 1952]. Historically, capacitation is defined as "the residential time of sperm required to bring it to the final functional maturation of sperm" [Chang, 1984]. This involves all the poorly understood morphological and biochemical processes involved in the maturation process. Capacitation mainly involves the remodeling of sperm plasma membrane (Yanagamachi, 1994; Visconti et al., 1995). This process includes redistribution of sperm membrane proteins, changes in the sperm intracellular ion concentration, increase in permeability of Ca^{2+} and bicarbonate ion and increase in the molar ration of phospholipids to cholesterol (Yanagamachi, 1994; Oliphant et al., 1985; Go & Wolf, 1983; Meizel, 1985; Langlais & Roberts, 1985). Capacitation is correlated with changes in the sperm metabolism, sperm cell volume, fluidity, motility, sperm cyclic nucleotide metabolism and sperm protein phosphorylation. Sperm capacitation can be achieved *in vitro* using appropriate salt solution containing electrolytes and metabolites in optimum concentration and serum albumin which altogether mimics environment of

oviductal fluid in many organisms. Even though the requirements and composition for sperm capacitation to happen is known, molecular basis and role of the factors involved and mechanism by which capacitation occurs is still enigmatic [Visconti et al., 1995].

1.1.5. Acrosome reaction

As described earlier, acrosome is the cap like structure present in the apical end of the sperm and acrosome reaction (AR) is a compulsory process the spermatozoa must undergo to attain the ability to penetrate through outer membranes of egg. AR can be defined as exocytosis of acrosome or fusion of acrsomal membrane with sperm membrane so that the contents of acrosome are released. In mammals, AR occurs in the ampulla of fallopian tube of female genital tract. AR is initiated by binding of sperm to the zona-pellucida of egg. This binding causes calcium influx into the cytosol, which triggers the exocytosis of acrosome, which concurrently results in the release of hydrolytic enzymes that help in dissolving egg protective coat. At the molecular level, the sperm-egg fusion is a carbohydrate mediated process. Even though many factors can trigger AR, ZP3 present on the egg surface is found to be the natural inducer of AR. The mode of this interaction is found to be though binding of sperm and sugar moiety (Lewis X-acid) on ZP3 [Pang et al., 2011]. This binding results in the activation of ion channels [Florman, 1994; Florman et al., 1998; Aranoult et al., 1996] and G-protein activation (especially G1 protein) [War et al., 1992, 1994]. Binding of ZP3 to sperm results in membrane depolarization and calcium influx [Aranoult et al., 1996; Lievano, 1996].

1.1.6. Fertilization

Fertilization can be defined as fusion of male and female gametes to develop an individual organism. In mammals, fertilization is the union of egg and sperm,

resulting in the formation of zygote. Fertilization occurs in the ampulla of fallopian tube. The capacitated but acrosome intact sperm interact with the zona pellucida of the egg loosely and reversibly, triggering acrosome reaction. In the later stage this interaction becomes rigid, species specific and irreversible. This results in the transfer of nucleus from sperm to egg where fusion of both nuclei occurs and results in the formation of zygote [Gwatkin, 1977; Wasserman, 1987; Yanagamachi, 1994]. The egg activates itself upon fusing with a single sperm and thereby changes the cell membrane to prevent fusion with any other spermatozoa [Wassermann, 1999].

1.2. Seminal plasma

Seminal plasma is the liquid entity of semen which is released along with sperm during ejaculation. Seminal plasma is a mixture of contents secreted from testis, epididymus, prostate and accessory sex glands such as seminal vesicle and bulbourethral gland. Seminal plasma contains both organic and inorganic entities such as metal ions, sugars, aminoacids, basic amines such as spermine, spermidine, putriscine and cadaverine and lipids. Proteins are the only high molecular weight biopolymers present in it, nucleic acids and polysaccharides are absent in it [Shivaji et al., 1990]. The seminal plasma provides a nutritive and protective medium for the spermatozoa during their journey through the female reproductive tract. The normal environment of the vagina is a hostile one for sperm cells, as it is very acidic (because of lactic acid produced by microflora), viscous, and guarded by immune cells. The components in the seminal plasma attempt to compensate for this hostile environment. These alkaline bases counteract and buffer the acidic environment of the vaginal canal, and protect DNA inside the sperm from acidic denaturation. It also contains decapacitation factors which prevent premature acrosome reaction. The metal ions present in the seminal plasma such as Ca^{2+} and Mg^{2+} play important role in maintaining basic pH during further stages of fertilization.

1.2.1. Bovine seminal plasma proteins

Four Acidic proteins secreted form the seminal vesicles constitute the major protein content of the bovine seminal plasma which are named as BSP-A1, BSP-A2, BSP-A3 and BSP-30kDa [Manjunath et al., 1987a &b; Chandonnet et al., 1990; Schiet et al., 1988]. The primary structure of all the above proteins shows high similarity [Seidah et al., 1987; Calvete et al., 1996a]. BSP-A1, A2 and A3 are small proteins with molecular weight ranging from 12-15 kDa, where as BSP-30 kDa as the name itself indicates is a 30 kDa protein [Manjunath et al., 1987a]. BSP-A1 and BSP-A2 have the same primary structure but differ in the extent of glycosylation and their inseparable mixture is known as PDC-109 [Esch et al., 1983]. All the four proteins are characterized by possessing two tandomly arranged repeating units of fibronectin type II (FnII) domains [Calvete et al., 1996a]. These proteins show affinity towards a variety of ligands such as choline phospholipids, sphingomyelin, heparin, HDL, LDL, gelatin, apolipoprotein A1 and different types of collagens (I, II, IV and V) [Desnoyer & Manjunath, 1992; Therien et al., 1995].

1.2.2. PDC-109: Structure and function

Protein containing N-termminal aspartic acid (D) and C-terminal cystein (C) with 109 amino acids, PDC-109 is the major protein of the bovine seminal plasma and is present at a very high concentration (15-20 mg/ml) in seminal plasma [Schiet at al., 1988]. During and upon ejaculation ~9.5 million PDC-109 molecules bind to the sperm surface at acrosome and some areas of mid piece [Calvete et al., 1994]. PDC-109 interaction with sperm membrane is mediated through specific interaction between PDC-109 and zwitterionic phospholipids containing choline head group [Desnoyer & Manjunath, 1992]. This binding lead to a process called ‘cholesterol efflux’ which is the removal of phospholipids mainly, choline phospholipids and

cholesterol, from the sperm plasma membrane. This process leads to a decrease in the cholesterol to phospholipids ratio in the sperm membrane [Therien at al., 1998; Moraeu et al., 1998].

Structurally, PDC-109 has an N-terminal stretch of 23 amino acid residues followed by two tandemly repeating fibronectin type-II (FnII) domains [Esch at al., 1983]. Single crystal X-ray analysis of PDC-109 complexed with PrC has showed that both binding sites are on the same face of the protein. Binding occurs mainly through cation-π interaction between the quaternary ammonium group of choline moiety and indole ring of a core tryptophan residue. In addition binding is stabilized by several hydrogen bonds between the phosphate group and tyrosine residues of PDC-109 [Wah et al., 2002].

Apart from showing affinity towards choline phospholipids, PDC-109 binds to a variety of molecules such as heparin, collagen, gelatin, high density lipoproteins, low density lipoproteins, apolipoprotein A1, fibrinogen, casein, glycosaminoglycans and α-, β- lactalbulins [Plante et al., 2015]. Among these their interaction with phospholipids is investigated in detail. EPR studies have revealed that selectivity towards choline phsopholipids is in the following order: phoshphatidyl choline ~ sphingomyelin > phosphatidic acid, phosphotidylglycerol ~ phosphotidylserine ~ androsterol > phosphatidylethanolamine > N-acyl phosphotidylethanolamine >> cholestane [Ramakrishnan et al., 2001]. Similar results were obtained from SPR measurements with association constants of interaction of PDC-109 with different lipids are in the order of DMPC > DMPG > DMPA > DMPE. The lipid specificity of PDC-109 towards choline phospholipids was shown to be because of faster association and slower dissociation compared to phospholipids containing other head groups [Thomas et al., 2003].

The sperm capacitation was found to occur through two different mechanisms, PDC-109-heparin induced and PDC-109-HDL induced capacitation [Manjunath & Therien, 2002]. Apart from binding to choline phospholipids on the sperm membrane and induce cholesterol efflux, PDC-109 has been implicated in various functions related to sperm activity and function. PDC-109 has been shown to be involved in the formation of oviductal reservoir as sperm containing PDC-109 was shown to bind to the oviductal epithelium [Ignotz et al., 2001]. PDC-109 was also found to regulate the cell volume of sperm in response to stress conditions [Sahin et al., 2009]. PDC-109 was also shown to protect sperm against cold shock during semen preservation [Barrios et al., 2005].

1.2.3. Chaperone like activity of PDC-109

1.2.3.1. Chaperones

Chaperones are proteins which assist in proper folding/ unfolding and assembly/ dissembly of other proteins but are not present in the protein machinery when the latter is performing their biological function [Lasky et al., 1978]. Chaperones are the second most abundant and highly important class of proteins in the cell. In physiological conditions, under both normal and stress conditions, chaperones protect newly synthesized polypeptides from misfolding and aggregation [Ellis, 1987; Horwitz, 1992; Hartl, 1996; Rajaraman et al., 1996; Kumar et al., 2005]. Several major classes of chaperone proteins are present in cells and in the extra cellular regions. They are Hsp100, Hsp90, Hsp70, Hsp60, Hsp40 and small heat shock proteins (sHsp).

Hsp100 or Clp are a highly conserved family of proteins present in almost all organisms. They have unfoldase activity and exist in hexamaric structures. Some of the members of this family like ClpA, ClpX etc. are associated with the serine

proteases and hence instead of typical refolding of the misfolded proteins they send the target proteins towards degradation pathway when they are beyond refolding condition [Glover et al., 1998].

Hsp90 family proteins are present in almost all living organisms, from bacteria to mammals. In mammals, Hsp90 exist in dimeric form and functions with a wide variety of co-chaperones [Prodromou et al., 1997]. Hsp90 chaperone action depends on the nature of substrate. If the substrate is in a confirmation that can be recognized by Hsp90 then in presence of co-chaperone and substrate is refolded in to active confirmation. In case where substrate cannot be recognized by Hsp90 then the substrate is directed towards degradation pathway [Wang et al., 2006].

Hsp70 family proteins are present in almost all the cell organelles indicating their importance in cell survival and growth. Hsp70 typically contains two domains. An highly conserved N-terminal domain which shows weak ATPase activity and a C-terminal end which is the peptide binding domain. Hsp70 requires J- domain proteins as co-chaperones to refold the substrate. Together chaperone and co-chaperone system covers the hydrophobic segments of the substrate causing reduction in intermolecular interaction thereby reducing the protein aggregation [Goloubinoff et al., 1999; Palleros et al., 1993].

Hsp60 is also called chaperonin because of its mediation in folding of different kind of proteins both *in vivo* and *in vitro* [Ellis and van der Vies, 1991]. These proteins are coded by heat inducible hsp60 gene. They have a characteristic oligomeric structure consisting of 14 subunits of 60 kDa monomers arranged in two heptameric rings stacked on top of each other [Hendrix, 1979; Hohn et al., 1979; Pushkin et al., 1982]. GroEL is the most famous protein of this family present in bacteria and regulates the assembly of bacteriophages [Georgopolous et al., 1973]. GroEL is an ATP dependent chaperone. In the absence of ATP it binds to the substrate and stabilizes it. When ATP is available, a stepwise release of substrate takes place, causing controlled

folding of the substrate [Langer et al., 1992a; Martin et al., 1991; van der Veis et al., 1992].

Hsp40 family is characterized by a conserved domain with 78 amino acid residues. This family consists of more than 100 proteins. These proteins interact with Hsp70 in the presence of ATP and acts as co-chaperones for Hsp70 [Laufen et al., 1998; Cyr, 1995]. Some proteins of this family such as DnaJ show chaperone activity independently [Hendershot et al., 1996]. A small conserved segment of 'His-Pro-Asp' is found to be responsible for the interaction of DnaJ with Hsp70 [Hill et al., 1995; Quian et al., 1996].

Small heat shock proteins (sHsp) along with α-crystallin family proteins consist of majority of extracellular heat shock proteins. They are typically large oligomeric structures of up to 1000kDa with monomers of 12-43 kDa proteins [Laufen et al., 1998; Sun & MacRae, 2005]. The oligomeric nature is found to be essential for exhibiting chaperone like activity. Another difference between this family and other Hsp's is that these proteins are ATP independent in nature [Horwitz, 1992]. They have no particular substrate specificity but shows high affinity towards partially unfolded intermediates. The proposed mechanism is sHsp's is that they coat the target protein through hydrophobic interactions and forms stable complexes and provide a reservoir for the other chaperone complexes to refold the substrate proteins [Lee et al., 1997].

1.2.3.2. Spermatozoa associated chaperones

In the male reproductive system several molecular chaperones have been identified. Most of them have different specificity towards somatic and germ line systems. Spermatozoa itself carries some chaperones such as DnaJB1, HspD1, HspE1 and HspA1A [Mitchell et al., 2007]. These chaperones are localized in the

mid piece and principal piece of the sperm tail. HspA1A was also detected in the equatorial region of the sperm along with HspCA. The locolization of these chaperones, HspA5 and HspH1 is not known so far and Tra1 was identified to be localized on the base part of the sperm head region. Even though the actual roles of these chaperones are not characterized so far, studies on HspA1A has shown that it is very crucial for fertilization as addition of anti HspA1A antibody has reduced the rate of fertilization [Spinaci et al., 2005].

1.2.3.3. Chaperone-like activity of PDC-109

As described above, even though sperm contains some chaperone machinery seminal plasma is completely devoid of them. Recently chaperone like activity of a major protein of bovine seminal plasma has been discovered [Sankhala & Swamy, 2010]. Several lines of evidence have indicatede that PDC-109 can act as a chaperone *in vitro*. PDC-109 was able to protect G6PD against heat stress condition and help it to retain its native confirmation even at high temperatures. Turbidimetric studies have shown that PDC-109 is able to solubulize aggregation prone proteins such as ADH, LDH and aldolase. Prevention of insulin fibrillation by PDC-109 is physiologically significance as some proteins of seminal plasma are found to be prone to amyloidogenesis. PDC-109 was able to refold the chaotrope induced unfolding of enzymes such as LDH. All these results indicate that PDC-109 can act as molecular chaperone, *in vitro*.

The chaperone-like activity of PDC-109 was found to be dependent on its polydisperse nature. PDC-109 exists mainly as a ~72 kDa oligomer in bovine seminal plasma. Addition of PrC or high ionic strength dissociates the oligomeric PDC-109 in to dimeric species [Gasset et al., 1997]. Presence of PrC or high ionic strength resulted in a loss of chaperone-like activity, indicating that polydispersity is

crucial for the CLA of PDC-109. PDC-109 complexed with lipid membranes show high chaperone like activity than PDC-109 alone. This is found to be because of high hydrophobicity exhibited by PDC-109/ lipid complex that PDC-109 alone. Presence of cholesterol in the lipid membrane has decreased the CLA [Sankhala et al., 2011]. This phenomenon can be attributed to the fact that cholesterol caused partial membrane rigidification or direct interaction with CARC domain of PDC-109 [Scolari et al., 2010]. These results strongly suggest that polydispersity and hydrophobicity are important factors for the CLA of PDC-109.

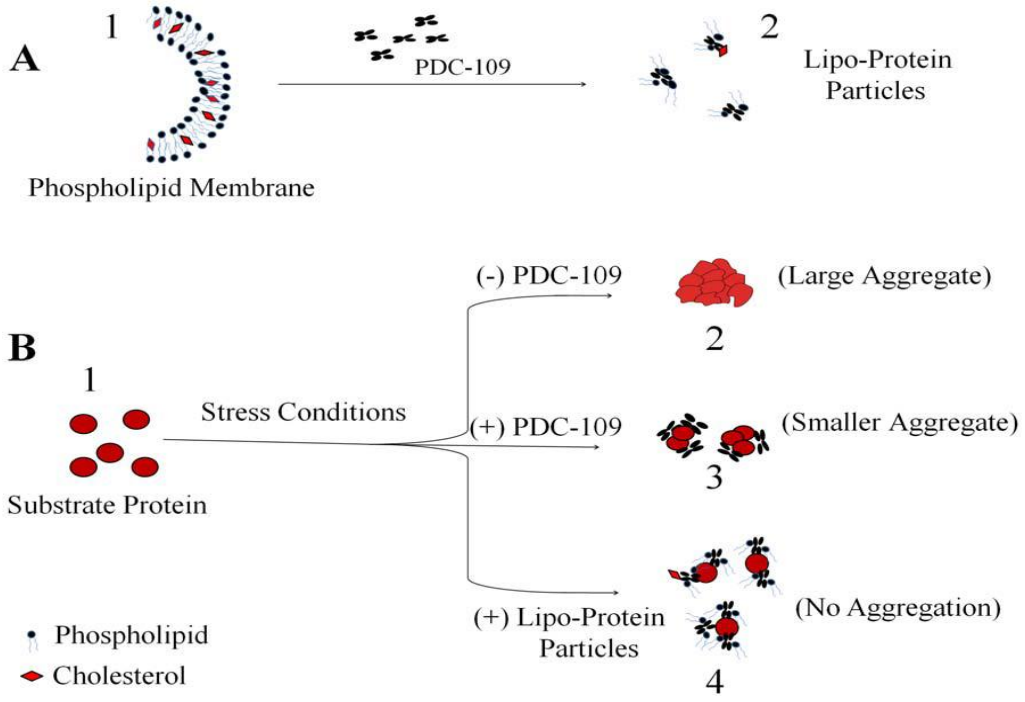


Fig. 1.2. A schematic model showing role of PDC-109 in sperm capacitation. A) PDC-109 induced cholesterol efflux. B) Chaperone like activity of PDC-109. (Taken from PLoS ONE 6(3): e17330. doi:10.1371/journal.pone.0017330)

1.3. Equine seminal plasma proteins

The protein content in equine (horse) seminal plasma is relatively low (10 mg/mL) compared to other mammals like bovine (up to 60 mg/mL). Equine seminal plasma proteins tend to form multi-protein aggregates of high molecular weight (~800 kDa) which are composed of low molecular weight proteins (11-30 kDa) [von Fellenberg et al., 1985]. The major proteins of horse seminal plasma were isolated by RP-HPLC and designated as HSP-1 to HSP-8 (Horse Seminal plasma Protein), based on their elution time/order. All these proteins have low molecular weight (14 to 30 kDa) and except HSP-4, all the proteins are bound to the equine sperm and can be isolated from the sperm extract [Calvete et al., 1994]. HSP-1 and HSP-2 belong to fibronectin type-II proteins. HSP-3 belongs to a family of proteins called cystein-rich secretory proteins (CRISP). HSP-4 is related to calcitonin gene like protein. HSP-6 and HSP-8 are isoforms of human kallikrein like proteins and have high similarity with human prostate specific antigen (PSA). HSP-7 belongs to a protein family called 'Spermadhesins', which are the major proteins in porcine seminal plasma containing a single CUB domain (complete β-sheet structure with conserved hydrophobic and aromatic residues). The N-terminal sequence of HSP-5 could not be related to any known family of proteins. HSP-1, HSP-2 and HSP-7 are the only known lipid binding proteins among the above stated. Even though all the proteins except HSP-4 are known to be present on the sperm head, their mode of anchorage is still unknown.

1.4. Horse seminal plasma protein-1 and -2 (HSP-1 and HSP-2)

Seminal FnII proteins are characterized by two or four tandemly arranged fibronectin type-II domains with N-terminal acidic stretches [Menard et al., 2003]. Short Fn-II proteins containing two Fn-II domains are the major proteins in seminal plasma of bull, goat, cattle and horse. In minor quantities, these proteins are also

present in porcine, ram, murine and human seminal plasma [Fan et al., 2006]. Long FnII proteins containing four fnII domains are present in human and equine seminal plasma and are termed SE-12 and EQ-12, respectively [Saalmann et al., 2001]. Because of their low expression and low abundance in the seminal plasma no lipid binding studies has been done on the long FnII proteins till date.

1.4.1. Structure of horse seminal plasma proteins, HSP-1/2

The primary structure of HSP-1 contains 121 amino acids, which structurally can be organized into four domains (AA'BB') [Calvete et al., 1995]. As described earlier, they contain two Fn-II domains designated as BB', with a free N-terminal flank containing two repeated units eachcontaining 16 amino acids, designated as A and A'. The N-terminal domains have two O-glycosylation sites each, containing sailylated Gal-GalNAc glycans. HSP-2 contains only one N-terminal 'A' domain hence has lower degree of glycosylation. Both proteins exist in different iso- and glycoforms, coded by a single gene, 'sp1' [Ekhlasi-Hundrieser et al., 2004]. HSP-1 and HSP-2 could not be separated under non-denaturing conditions; hence their mixture is referred to as HSP-1/2. Secondary structure of HSP-1/2 is very similar to that of PDC-109 with no major changes in the far UV or near UV CD spectra on addition of PrC [Anbazhagan & Swamy, 2005; Sankhala et al., 2012]. However, PrC binding stabilizes the protein structure against thermal denaturation in a concentration dependent manner, with the unfolding temperature increasing from 49^{o}C in the absence of PrC, whereas in the presence of 10 and 20 mM PrC the unfolding temperature increases to 57 and 60^{o}C, respectively [Sankhala et al, 2012]. HSP-1/2 has a complex quaternary structure. It exists as a mixture of monomer and ~90 kDa oligomer, unlike PDC-109 which predominantly exists as an oligomeric form [Calvete et al., 1997]. This polydisperse nature was mainly attributed to the difference in glycosylation of HSP-1/2 [Calvete et al., 1995]. Similar to PDC-109 it

was shown that on addition of PrC the ~90 kDa oligomers are reduced to monomer or dimer [Calvete et al., 1997].

1.5. Lipids

Lipids are one of the four major classes of biomolecules in the living systems others, the being proteins, DNA and RNA. They are the main constituents of the membranous structures covering the cells and intracellular organelles. As they seperate the cells from outer environment and organelles in the cell from each other, they are of great importance. Other than acting as structural components, lipids are used for energy storage and some lipids and their derivatives act as hormones and vitamins. Structurally lipids are the small organic molecules which are mostly not soluble in water but are soluble in organic solvents such as acetone, chloroform, ether etc. This solubility in organic solvents is mainly because of their hydrophobic nature. Typically lipds contain a hydrophilic head connected to two hydrophobic chains most of which are either alkyl or acyl chains. Based on their structure lipids are classified in to two major classes. 1) **simple lipids**, which cannot be hydrolyzed and hence are basic functional units in most of the membranous structures such as cholesterol and other steroids. 2) **complex lipids**, which yield smaller molecules such as hydrophilic group and hydrophobic tail separately, such as fats and waxes.

1.5.1. Types of lipids

All the organisms contain one or the other forms of membranous structures. The membrane mainly consists of lipids and proteins with lipids. The most accepted model of the membrane is the ‘Fluid-Mosaic model’ proposed by Singer and Nicolson in 1972. According to this model, membrane is a non-polar lipid bilayer which is fluidic in nature because of the saturated and unsaturated fatty acids present in it. The mosaic nature of the membrane comes from the presence of various other

molecules such as proteins, sterols etc [Singer & Nicolson, 1972; Singer, 1974]. This model also indicates the dynamic nature of the lipids in the membrane due to diffusion through two-dimensional surface of the membrane and lipid 'flip-flop' i.e. jumping of lipid from one side to the other side (inner leaflet to outer leaflet). Membrane mostly consists of three major types of lipid: glycolipids, sterols and phospholipids.

Phospholipids: Phospholipids constitute the major portion of lipid membranes. These are amphiphilic in nature, contains negatively charged phosphate moiety in the head group and two hydrocarbon chains as the hydrophobic tail part. Due to high hydrophobicity of the tail part they segregate towards each other creating a non-polar environment in the membrane with phosphate group facing the outer hydrophilic environment.

Phosphoglycerides: These are derived from glycerol and contain a phosphate group. Phosphatidic acid (PA), the simplest phosphoglyceride with two hydroxyls of glycerol attached to fatty acyl chain and the other hydroxyl attached to the phosphate group. PA is absent in cell membranes, instead derivatives of PA are found as commonly occurring lipids in the cell membranes. These derivatives based on the additional group present can be called as phosphotidylcholine, phosphotidylserine, phosphotidylethanolamine, phosphotidylinisitol and phosphotidylglycerol. Cardiolipin or diphosphatidylglycerol is a four chain lipid present in the heart tissue. Structures of these molecules are shown in Fig. 1.3.

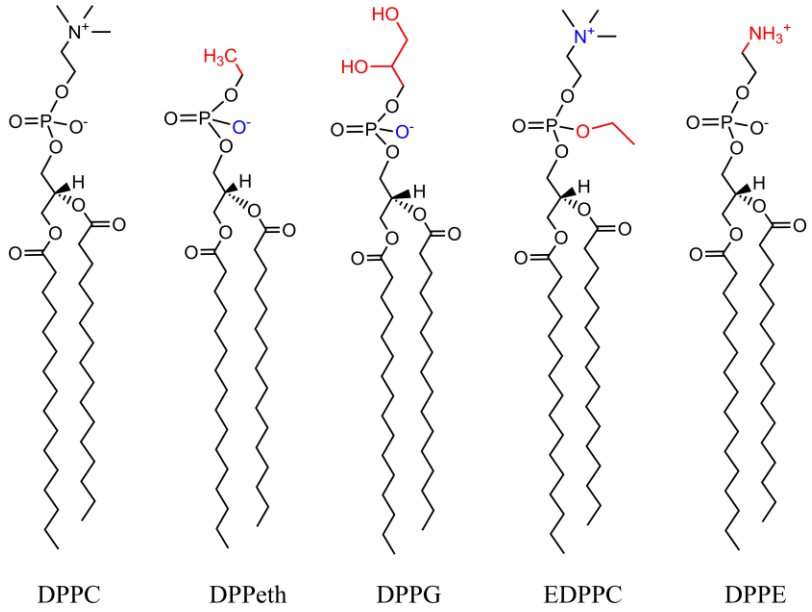


Fig. 1.3. Structures of different phospholipids (Taken from http://.doi:0.5772/51882)

Sphingolipids, contains a sphingosine as the back bone, which contains amino group at 2-hydroxy position of glycerol moiety and unsaturated (4C-5C) long alkyl chain attached to the 3rd carbon. Based on the additional groups attached to this back bone the sphingolipids are further classified into ceramides (N-acylated shpingosines), sphingomyelins, phosphocholine or phosphoethanolamine attached to 1-hydroxy position of ceramide. If a sinlge carbohydrate molecule (a monosaccharide) is attached to ceramide they are called as cerebrocides. Ceremides with at least twosugar molecules attached to them with at least one sialic acid are called gangliosides.

Sterols, are compact, polycyclic (mostly 5 or 6 membered ring), rigid, hydrophobic molecules with one polar hydroxyl group. These are found in microbial, plant and animal kingdoms with cholesterol being the most commonly occurring one. Cholesterol constitutes ~30% of the plasma membrane lipids in animals, but is also

present in lysosomes, endosomes and golgi complex. Sterols play an important role in maintaining order, fluidity/rigidity of the cell and cell organelle membranes.

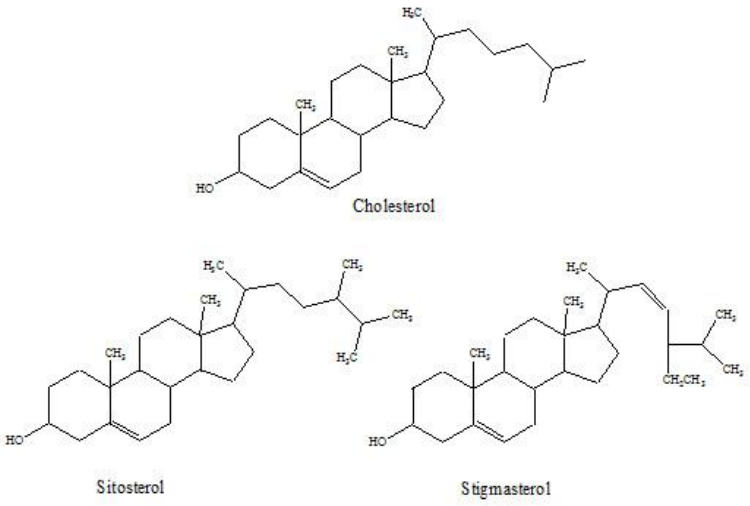


Fig. 1.4. Structures of different sterols

1.5.2. Self assembly of lipids in excess water

when amphiphilic molecules such as phospholipids are mixed with excess water, specific structures such as micelles, bilayers, liposomes or vesicles, inverted micelles etc are formed to minimize the exposure of the hydrophobic part to water. The formation of the above mentioned supra molecular assemblies depends on the critical packing parameter which in turn depends on the size and shape of the hydrophilic and hydrophobic parts of lipid. The formation also depends on the environmental factors such as temperature, pressure, pH and ionic strength.

Micelles are the aggregates of the lipids with the hydrophilic part of the amphiphile oriented towards the solvent interface and the hydrophobic tail part oriented away from the polar phase. Micelles may take several forms such as disks, rods, distorted spheres etc. depending on conditions and composition of the lipid. Reverse micelles

are formed in non-polar solvents such as benzene when amphiphile clusters around a small water droplets in the system.

Monolayers are formed spontaneously when polar lipids are added to aqueous solution. They are one layer thick with hydrocarbon tails of the polar lipid exposed to air and the hydrophilic heads in contact with the aqueous phase.

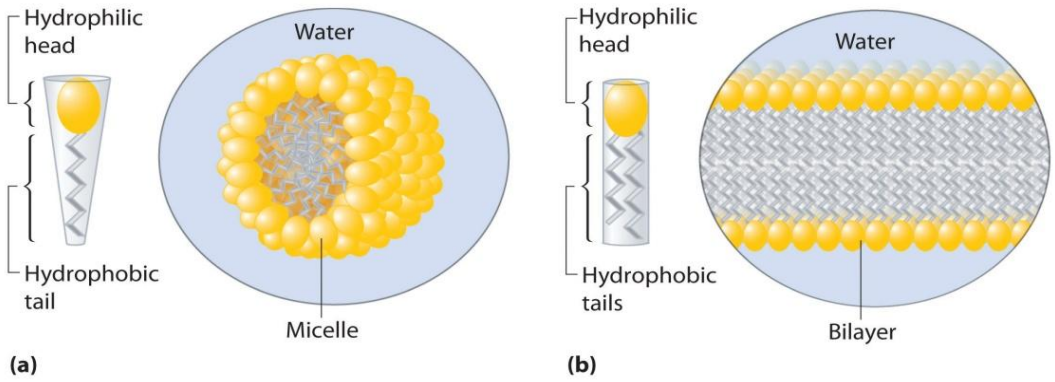


Fig. 1.5. Schematic representation of micelles and bilayer (Taken from http://chemwiki.ucdavis.edu/Wikitexts/University of California Davis/UCD Chem 2B/ UCD Chem 2B%3A Larsen/Unit II%3A States of Matter /Solutions /13.6%3A)

Bilayers and multilayers are formed by phospholipids and other amphiphiles when suspended in aqueous media and separate two or more aqueous compartments. Phospholipid bilayers are ~ 6-7 nm thick depending on the nature of the fatty acid constituting the lipid. Bilayers contain hydrocarbon tails extending inward from the two surfaces of the water to form continuous inner hydrocarbon core and the hydrophilic heads face towards aqueous phases.

Liposomes are spherical vesicular structures constaining phospholipid alone or mixed with cholesterol or any other lipid bilayer. Liposomes contain an encapsulated aqueous solution inside the hydrophobic membrane. Any chemical which is hydrophobic in nature can be dissolved into the membrane. Liposomes are usually

made by sonicating the membrane bilayer or multilayers in water for extended periods of time. In pharmaceutical applications liposomes are used as carriers for delivering vaccines, enzymes, hormones, genes and drugs.

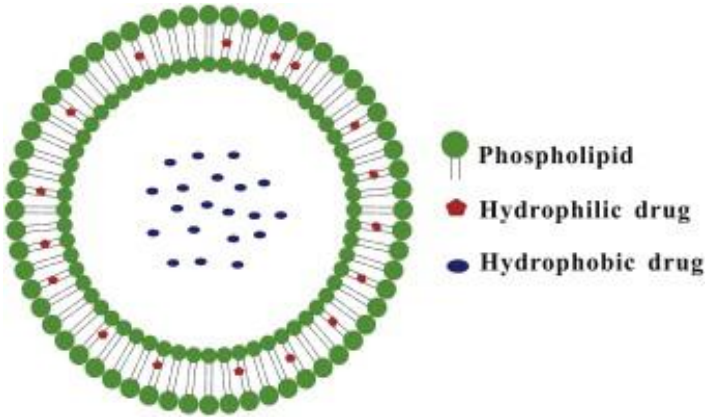


Fig. 1.6. Schematic picture of a liposome used for drug delivery

(Taken from http://dx.doi.org/10.1016/j.ajps.2014.09.004)

1.5.3. Interaction of HSP-1/2 with lipid membranes/vesicles

Interaction of HSP-1/2 with phospholipid membranes was investigated in a study by gruebe et al.[2004] using fluorescence and EPR spectroscopy. the important features of the interaction are very similar to those of PDC-109-phospholipid interaction. Similar to PDC-109, intrinsic fluorescence spectrum of HSP-1/2 was blue shifted upon interaction with choline phospholipids. Similar to PDC-109, SP-1/2 has specificity for phospholipids with the phosphocholine headgroup. Upon binding to lipid vesicles, the protein intercalates into the hydrophobic membrane core, resulting in a rigidification of the lipid phase and, at higher concentration results in the perturbation of membrane structure. However, compared with PDC-109, the impact of SP-1/2 on membranes is less intense in that the degree of protein-mediated immobilization of lipids was lower. Furthermore, SP-1/2 was not able to

extract lipids from human red blood cells to the same extent indicating species specific differences.'

From the studies of interaction of HSP-1/2 with spin labeled cholesterol analogues using EPR Gruebe et al. [2004] has observed that a higher protein to lipid ration is required to cause any rigidification of lipid membrane and was able to cause membrane perturbation resulting in the exposure of the inner leaf let of the liposome interacting with HSP-1/2.

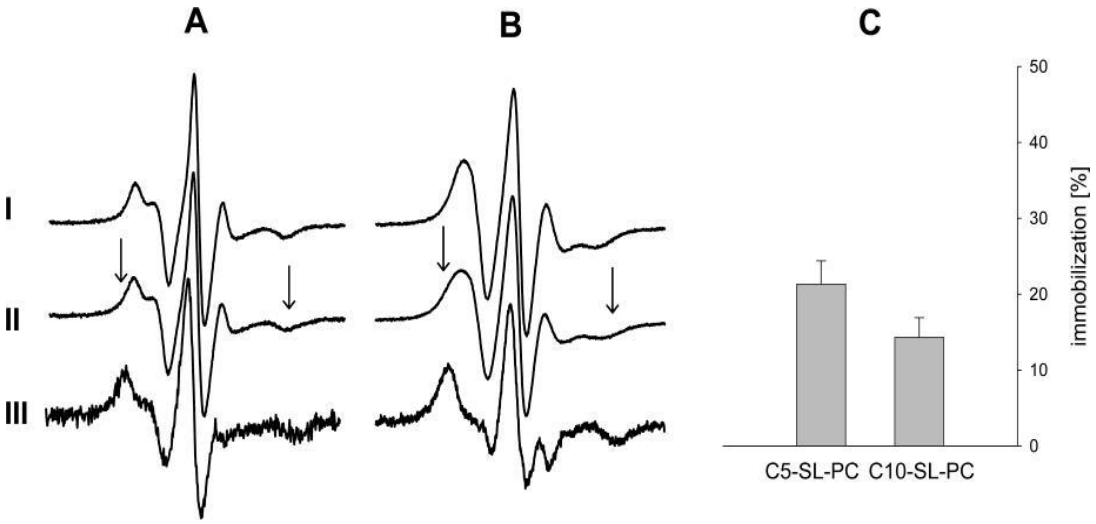


Fig. 1.7. Influence of SP-1/2 on long-chain spin-labeled PC incorporated into both leaflets of PC LUV. PC LUV (2 mM) was labeled with 50 *u*M C5-SL-PC (A) and C10-SL-PC (B) as described in Experimental Procedures. ESR spectra of labeled vesicles were recorded at 25 °C in the absence (I) and in the presence (II) of SP-1/2 (L/P). (B).The immobilized component observed in the presence of the protein (see arrows) was extracted by spectra subtraction, i.e., spectra II - I, yielding spectra III. (C)Spectra were recorded as described above but at L/P =10(Taken from http://pubs.acs.org/ doi/abs/10.1021/bi035647l)

1.6. Oxidative stress in reproduction

Infertility because of abnormalities in male reproductive system occurs at least in 50% of the total cases. Most of these are because of the "alteration in sperm concentration, motility, and/or morphology is present in at least one sample of two sperm analyses, collected 1 to 4 weeks apart" [Trussell, 2013; WHO, 2010]. Oxidative stress (OS) is found to be one of the major and plausible reason for the infertility. Oxidative stress is a condition in which imbalance occurs between the reactive oxygen species (ROS) producing systems and ROS scavenging systems in such a way that damage occurs to the total system. In general, spermatozoa are equipped with antioxidant machinery to tackle the OS, but under abnormal pathological conditions ROS production exceeds the antioxidant machinery capacity or antioxidant capacity itself decreases, creating a situation of OS [Hampl, 2012; Henkel, 2011].

1.6.1. ROS generation in sperm and seminal plasma

In active spermatozoa, electron leakage is observed because of the ROS. The generation of ROS in sperm occurs mainly via 2 mechanisms. one being NADP-oxidase system operating at plasma membrane of spermatozoa and the other one is the NAD dependent oxido-reductase system, which serves as a source of energy at the mitochondrial region of the spermatozoa. In human spermatozoa, superoxide ion is found to be the major ROS generated, which along with H_2O_2 in the presence of metal ions such as iron, copper, cobalt etc. undergoes Heber-Weiss reaction producing hydroxyl radical ($OH^{-\cdot}$), which is found to be pathological to the sperm, hindering their morphology, integrity and most importantly function [Henkel, 2011; Sikka, 2001]. In case of seminal plasma, ROS are produced from both endogenous and exogenous sources. In human semen, sperm cells and other cells from different

stages of sperm meturation process (spermatogenesis) are present along with epithelial cells and leukocytes. Among these cells, immature sperm cells and the defense cells such as neutrophils and macrophages are the main source of the endogenous production of ROS. Life style choices and factors such as smoking and drinking, excessive use of drugs are the main source of exogenous source of ROS. Environmental factors such as exposure to radiation and toxins are found to contribute to the production of ROS [Gharagozloo & Aitken, 2011; Esteves, 2002].

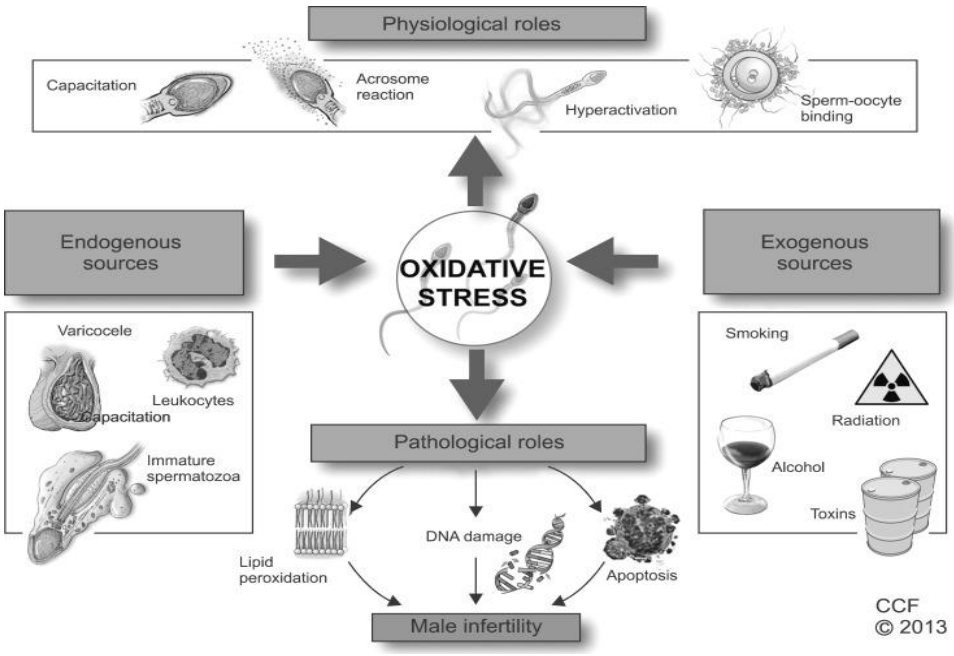


Fig. 1.8. Oxidative stress in male reproductive system.

(Taken from doi:10.5534/wjmh.2014.32.1.1)

Production of ROS is said to be of physiological significance as the ROS signaling pathway was found to regulate capacitation in an indirect manner [de Lamirande & O'Flaherty, 2008]. Hyper-activation of spermatozoa, which is essential for sperm motility and fertilization and acrosome reaction are also found to be

correlated with the ROS generation in sperm [Saurez, 2008]. Even though the generation of ROS in limited quantities is found to be useful, any imbalance in the ROS production and regulation is found to be pathological for spermatozoa. Depending on the nature, amount, and duration of the ROS insult, these defects cause significant damage to biomolecules such as lipids, proteins, nucleic acids, and sugars. Two of the main damaging cascades caused by ROS to spermatozoa are lipid peroxidation and DNA damage [Agarwal & Prabhakaran, 2005].

To counter the oxidative stress, sperm and seminal plasma contain two kinds of anti-oxidants. They are enzymatic and non-enzymatic anti-oxidants. Enzymatic anti-oxidants are naturally present in the system and they constitute mainly three enzymes viz. super oxide dismutase (SOD), catalase and glutathione reductase (GSH). Some non enzymatic anti-oxidants include vitamins C, E, and B, carotenoids, carnitines, cysteines, pentoxifylline, metals, taurine, hypotaurine and albumin [Lampiao, 2012; Sharma & Agarwal, 1996].

Table: 1.1. Anti-oxidants present in mammalian seminal plasma and their mode of action. (Taken from Agarwal et al., 2014, doi:10.5534/wjmh.2014.32.1.1)

Antioxidant	Mechanism of action	Effect
GSH/GPX	Scavenges for free radicals	Prevents lipid peroxidation and improves sperm membrane characteristics
Superoxide dismutase	Neutralizes superoxide anions	Prevents lipid peroxidation
Catalase	Breaks H_2O_2 down into H_2O and O_2	Prevents lipid peroxidation
Vitamin E	Neutralizes free radicals	Prevents lipid peroxidation and improves activity of other antioxidants
Vitamin C	Neutralizes free radicals	Protects viability and motility
Carnitine	Neutralizes free radicals and acts as an energy source	Prevents lipid peroxidation and DNA damage
Carotenoids	Quenches singlet molecular oxygen	Prevents lipid peroxidation
Cysteines	Increases the amount of GSH synthesized	Prevents lipid peroxidation
Pentoxifylline	Prevents cAMP breakdown and suppresses the synthesis of pro-inflammatory factors	Prevents lipid peroxidation

1.7. Objectives and major finds of the current study

Sperm capacitation is an important event that must occur for the final maturation of spermatozoa to participate in fertilization. Even though capacitation is known for last several decades, molecular basis of this process is not clearly known/understood [Chang, 1951; Austin, 1952]. Moreover, the significant differences observed between the steps involved in sperm capacitation appear to be species specific and cannot be generalized. Hence it is important to study each and individual species in order to obtain the complete picture of the capacitation process in mammals and factors involved in it.

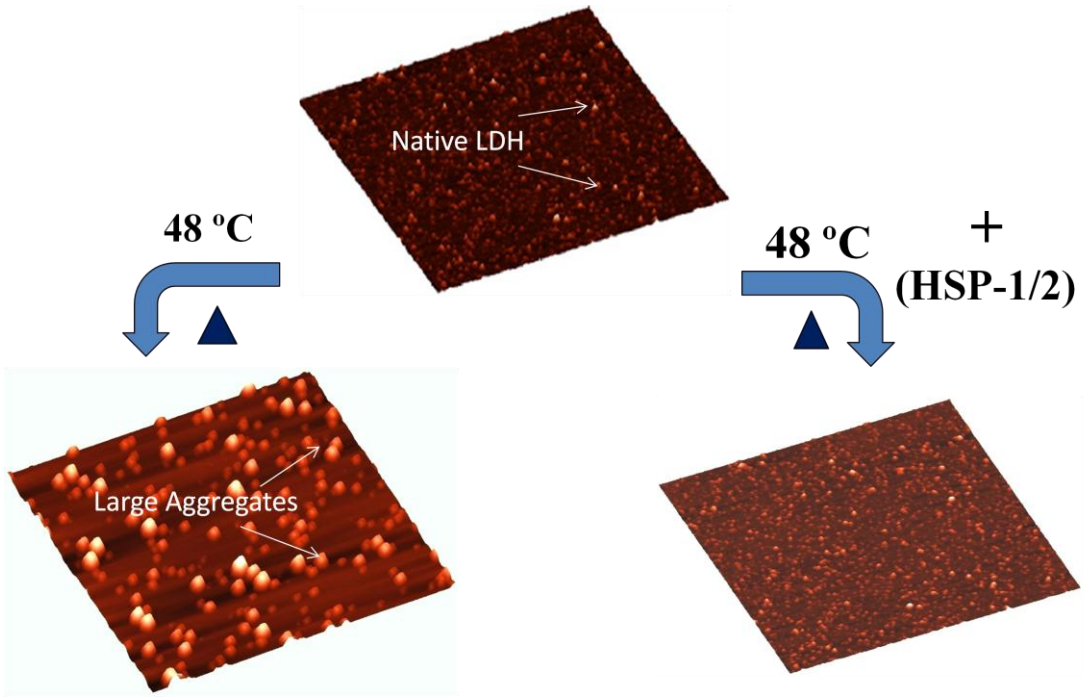
Recent studies have shown that the major bovine seminal plasma protein, PDC-109 exhibits chaperone like activity. Various line of evidence was given to show that PDC-109 is able to protect target enzymes against chemical and thermal stress condition. Inhibition of insulin fibrillation by PDC-109 indicates that it can prevent the non-specific aggregation of target polypeptides which are in both amorphous and fibrillar in nature [Rajeshwar & Swamy, 2010]. These results are significant considering that the 'traditional' hsp's (Heat Shock Proteins) are absent in the mammalian seminal plasma. Hence finding of a chaperone in a system such as seminal plasma which is prone to stress conditions is of greater significance. It is also shown that membrane binding which is the physiological role of PDC-109 increases the CLA of PDC-109 due to increase in hydrophobicity of the whole system [Shankala et al., 2011].

FnII proteins like PDC-109 are present in most of the mammalian seminal plasma such as horse, ram, bison, human, rat, porcine and goat [Plante et al., 2015]. Hence establishing the functional roles of these proteins in the respective

reproductive system is of considerable importance because the expression levels of these proteins vary from species to species indicating different physiological roles. In view of this, in the present thesis entitled "**Biochemical and Biophysical Characterization of Chaperone-like Activity and Lipid Binding to the Major Protein of Horse Seminal Plasma, HSP-1/2"** we tried of address the following problems/questions.

- Does HSP-1/2,similar to PDC-109 exhibit chaperone-like activity and protective non-specific target enzymes/proteins against thermal and chemical stress conditions? If it does show chaperone like activity, what are the factors responsible for exhibiting the CLA?
- Oxidative stress is an important problem for spermatozoa abnormality causing reduced fertility rates. Hence it is important to examine If HSP-1/2 can act as chaperone under oxidative stress and afford protection to target proteins in similar way to that of against thermal stress.
- HSP-1/2 interaction with choline phospholipids is of physiological significance as this interaction is found to be the molecular basis for *cholesterol efflux*. Except for study by Greube et al., [2004] no detailed study of this interaction is done. Hence HSP-1/2 interaction with phospholipids and chemical unfolding studies on HSP-1/2 are investigated.
- Further Experiments were directed to investigate the effect of HSP-1/2 on morphology and integrity on model cell and supported membranes. Effect of various conditions such as effect of ligands and pH was carried out and the results have shown that HSP-1/2 dual functionality (membrane destabilization and chaperone like activity) is regulated by a pH switch.

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



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* These two authors has contributed equally to this study

Biochem. Biophys. Res. Commun. **2012**, 427, 18-23

Chapter 2

2.1. Summary

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.

Chapter 2

2.2. 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 [Yanagimachi, 1994; Shivaji et al., 1990]. 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 [Desnoyers & Manjunath, 1992; Töpfer-Petersen, 1999; Gwathmey et al., 2003] 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 [Töpfer-Petersen et al., 2005]. Among the FnII proteins, the major protein of bovine seminal plasma, PDC-109, has been studied in great detail [Manjunath & Therein, 2002; Swamy, 2004; Anbazhagan et al., 2011; Tannert et al., 2007, Sankhala et al., 2011]. PDC-109 binds specifically to phospholipids containing choline head group, such as phosphatidylcholine and sphingomyelin [Desnoyers & Manjunath, 1992; Anbazhagan et al., 2011; Ramakrishnan et al., 2001; Greube et al., 2001; Swamy et al., 2002; Thomas et al., 2003].

Proteins homologous to PDC-109 have also been identified in several other mammals [Fan et al., 2006]. 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 [Calvete et al., 1994, 1995a & b]. 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 [Sankhala & Swamy, 2010]. 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 [Greube et al., 2004]. The results obtained indicate that similar to PDC-109, HSP-1/2 show greater affinity for choline phospholipids. The interaction of HSP-1/2 with phospholipid membranes and heparin appears to be physiologically significant in view of their involvement in sperm capacitation.

Recently work has 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 [Sankhala & Swamy, 2010; Sankhala et al., 2011]. Since HSP-1/2 shares significant homology with PDC-109 and exists in polydisperse oligomeric states [Calvete et al., 1995a ,1997], 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; therefore establishment of their presence in other mammalian species including humans is of great interest.

2.3. Materials and methods

2.3.1. Materials

Phosphorylcholine chloride calcium salt, glucose-6-phosphate dehydrogenase (from *Leuconostoc mesenteroides*), insulin (form bovine pancreas), glucose-6-phosphate and heparin-agarose type I beads were obtained from Sigma (St. Louis, MO, USA). *p*-Aminophenyl phosphorylcholine-agarose column was obtained from Pierce Chemical Co. (Oakville, Ontario, Canada). Lactate dehydrogense, alcohol dehydrogenase, nicotinamide adenine dinucleotide phosphate, tris base and other

chemicals were purchased from local suppliers and were of the highest purity available.

2.3.2. Purification of HSP-1/2

HSP-1/2 was purified by affinity chromatography on heparin agarose and *p*-aminophenyl phosphorylcholine-agarose, followed by reverse phase HPLC on a C-18 column [Calvete et al., 1994, 1997]. Briefly, stallion seminal plasma was separated from spermatozoa by centrifugation of the semen at 1500 × g for 15 min, and the supernatant obtained was further clarified by centrifugation at 6000 × g for 10 min at 4°C. About 50 mL of the clear seminal plasma was applied to a heparin-agarose column pre-equilibrated with 50 mM tris buffer, pH 7.4, containing 0.15 M NaCl and 5 mM EDTA (TBS). After washing with the same buffer till the column effluent showed an absorbance below 0.01, the bound proteins were eluted with the same buffer containing 20 mM phosphorylcholine. The eluted protein fraction was extensively dialyzed against TBS and applied to a *p*-aminophenylphosphorylcholine column. After extensive washing with the same buffer, the bound proteins were eluted with TBS containing 10 mM phosphoryl choline. Further separation of individual proteins in the bound fraction was done by reverse phase HPLC (RP-HPLC) using a Luna RP-100 C_{18} column (25×4.6, 5µm particle size) (Phenomenex, California, USA). The column was pre-equilibrated with a mixture of 0.1% trifluoroacetic acid in water (solution A, 75%) and acetonitrile (solution B, 25%). A flow rate of 1 mL/ml was maintained throughout. After sample loading, the column was run first isocratically with 25% B for 5 min, followed by a gradient of 25-30% of B for 5 min and 30-50% for 70 min. Peaks were collected manually and purity of the proteins was checked with 15% sodium dodecyl sulphate polyacrylamide gel electrophoresis [Lamelle, 1970].

2.3.3. 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 minutes with fixed concentrations of PrC before the temperature scans were performed.

2.3.4. 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://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.

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

Chaperone activity was assayed as described previously [Sankhala & Swamy, 2010] 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 minutes 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 minutes with different concentrations of PrC.

2.3.6. G6PD activity assay

G6PD activity was assayed by a spectrophotometric method essentially as described earlier for PDC-109 [Kumar et al., 2005]. 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 30 minutes 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.

2.3.7. Insulin aggregation assay

Insulin aggregation assay was performed as described earlier [Farahbakhsh et al., 1995]. 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 (1M 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 hour. Aggregation of native insulin was taken as 100% and percent aggregation of the remaining samples was calculated with respect to it.

2.3.8. 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 at 48°C in a heating bath for 20 minutes 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 minutes, 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 [Sankhala & Swamy, 2010].

2.4. Results and Discussion

2.4.1. Secondary 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. 2.1A. 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. 2.1B). These spectral features are similar to those of PDC-109 [Anbazhagan & Swamy, 2005, Gasset et al., 1997]. 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 data set [Gasset et al., 1997].

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. 2.1C). 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 (curve 1, Fig. 2.1C). In the presence of 10 mM PrC the midpoint of the transition shifts to ~57ºC (curve 2), whereas increasing the PrC concentration to 20 mM shifts the transition midpoint to ~60ºC (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 [Gasset et al., 1997].

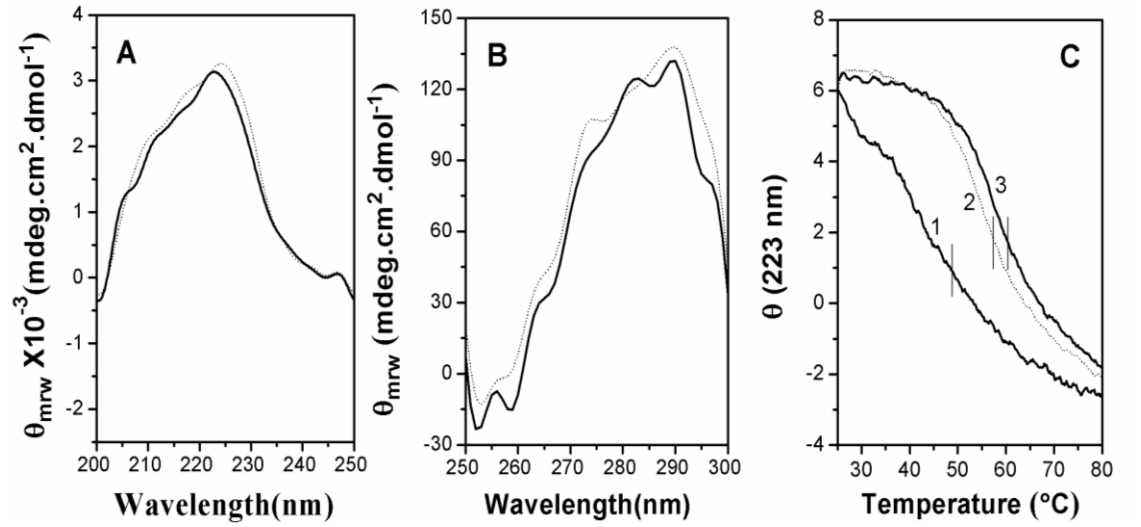


Fig. 2.1. Circular dichroism studies of HSP-1/2. (A) Far-UV CD spectra of HSP-1/2 alone (solid line) and in the presence of 20 mM PrC (dashed line), **(B)** Near-UV CD spectra of HSP-1/2 in TBS. **(C)** PrC induced thermal stability of HSP. Thermal scans of the protein (0.12 mg/mL) in the absence and 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.

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 [Wah et al., 2002]. 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. 2.2C). 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 Table 2.1.

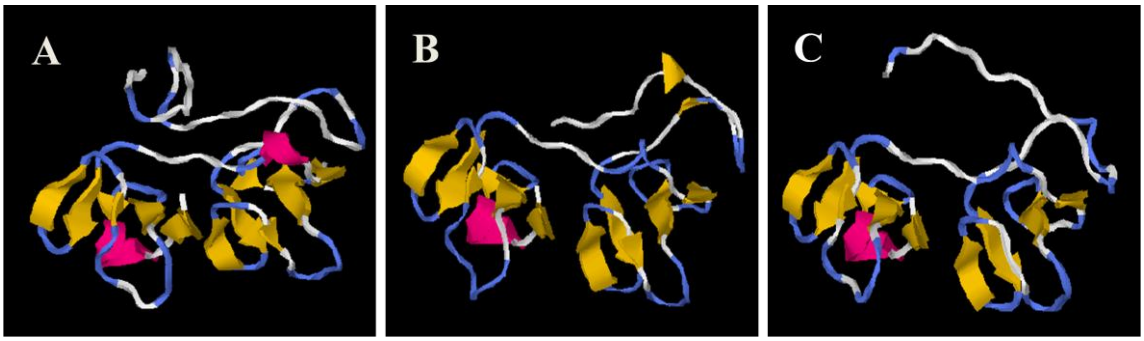


Fig. 2.2. 3-Dimensional structures of HSP-1 (A), HSP-2 (B) and PDC-109 (C) generated by I-TASSER server.

From the data presented in table 2.1. 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.

Table 2.1. Secondary structure of HSP-1, HSP-2 and PDC-109 estimated from computational modeling using the I-TASSER server. Secondary structure of PDC-109 determined from the crystal structure is also given for comparison.

Protein	α-helix	β-sheet	β-turns + unordered structures
HSP-1	5.8	25.0	69.1
HSP-2	3.8	27.6	68.5
PDC-109 (I-TASSER model)	3.7	23.8	72.4
PDC-109 (crystal structure)	9.2	22.9	67.8

3.4.2. Chaperone like activity of HSP-1/2

3.4.2.1. 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. 2.4A. 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 is given in Fig. 2.4B. HSP-1/2 exhibited similar chaperone-like activity against LDH also (Fig. 2.3). These results strongly suggest that HSP-1/2 protects target proteins from thermal denaturation, thus exhibiting CLA.

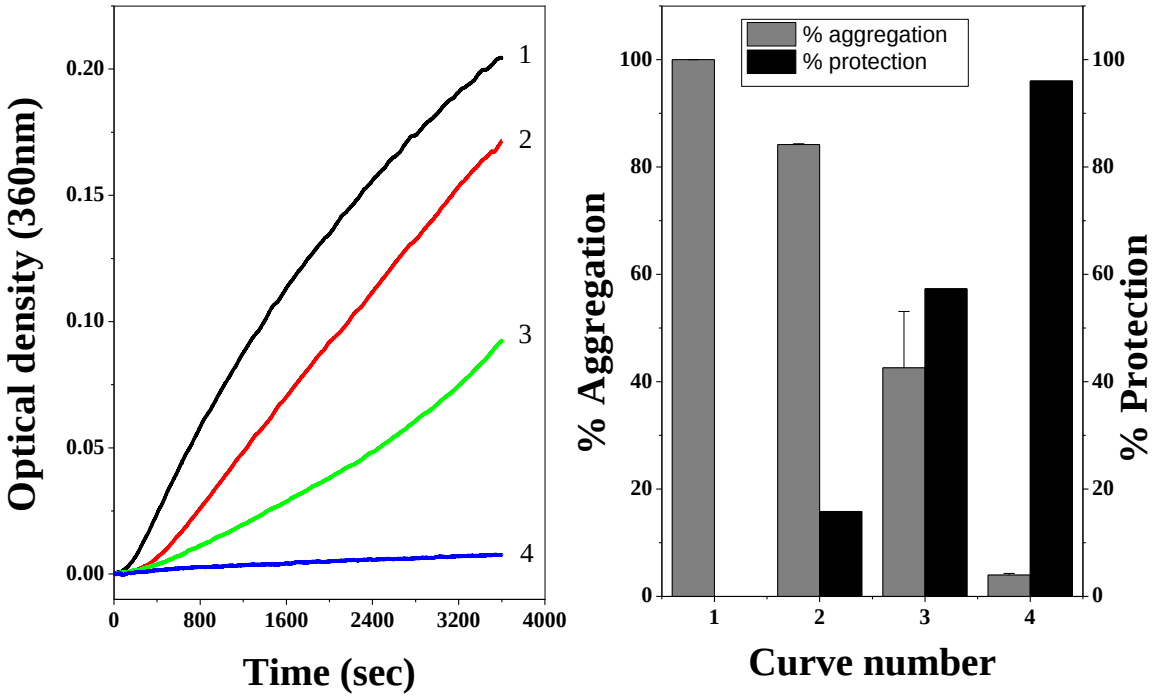


Fig. 2.3. Chaperone-like activity of HSP-1/2. A) Prevention of aggregation of LDH (0.075 mg/ml) by HSP-1/2. Aggregation profiles of (1) LDH at 49°C, (2) LDH + 0.05 mg/ml HSP-1/2, (3) ADH + 0.015 mg/ml HSP-1/2 and (4) LDH+0.01mg/ml HSP-1/2 are shown. **B)** Bar diagram representing percent aggregation (black bars) and protection (gray bars) of LDH by HSP-1/2 at different concentrations.

3.4.2.2. 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-1/2. When incubated at 45°C for 15 minutes G6PD lost about 50% of its

activity (Fig. 2.4C, 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-1/2 at 4ºC 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 lends further support to the above interpretation that this protein exhibits chaperone-like behavior.

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

Aggregation profiles observed with insulin upon incubation with DTT are shown in Fig. 2.4E. 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. 2.4E 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. 2.4F. These results indicate that HSP-1/2 protects insulin from aggregation induced by reducing agents such as DTT.

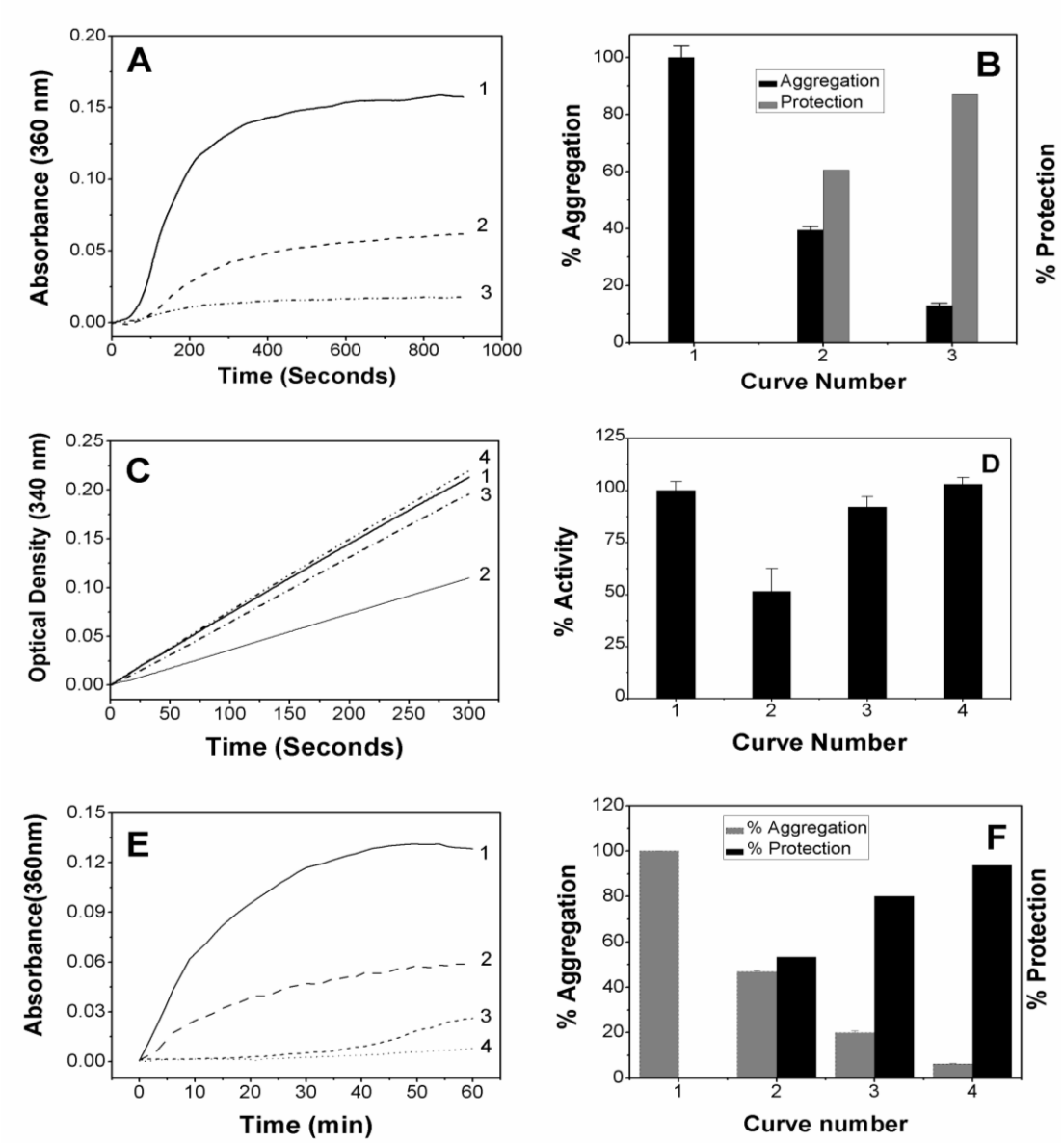


Fig. 2.4. Chaperone-like activity of HSP-1/2. A) Prevention of aggregation of ADH (0.05 mg/mL) by HSP-1/2. Aggregation profiles of (1) ADH at 48°C, (2) ADH + 0.01 mg/mL HSP-1/2 and (3) ADH + 0.025 mg/mL HSP-1/2 are shown. **B)** Bar diagram representing percent aggregation (black bars) and protection (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 seconds (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, (4) insulin+0.3 mg/mL HSP-1/2. **F**) Bar diagram representing percent aggregation (black bars) and protection (grey bars) of insulin by HSP-1/2 from panel **E**.

2.4.2.4. 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 the results obtained with LDH are presented in Fig. 2.5. AFM images of LDH obtained under native conditions show a uniform size-distribution with small particles (Fig. 2.5A). Upon incubation at 48°C the protein yields fairly large aggregates (Fig. 2.5B).

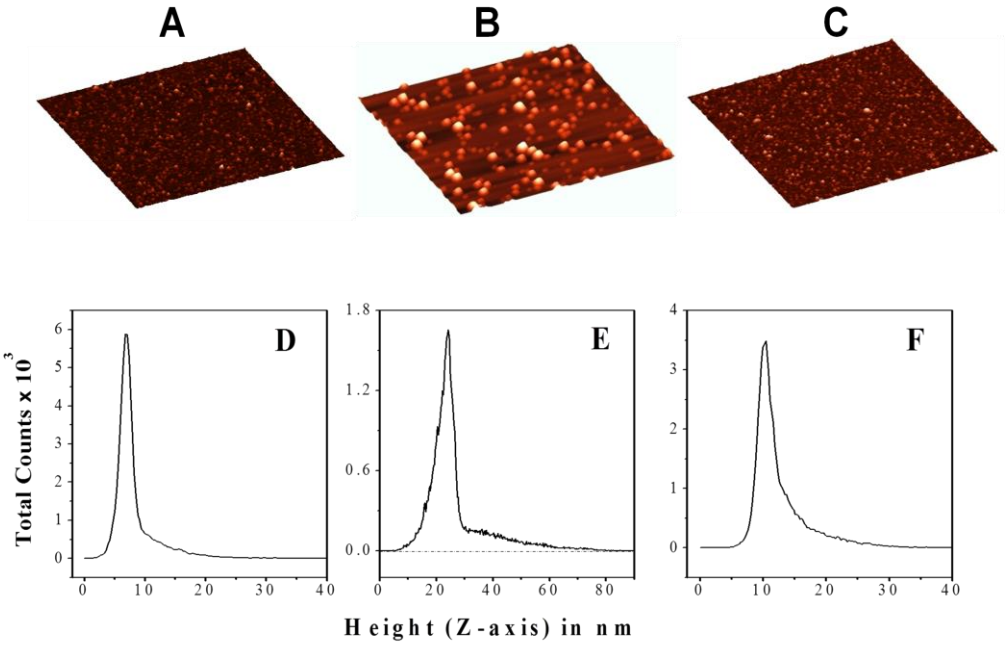


Fig. 2.5. Prevention of thermal aggregation of LDH by HSP-1/2. AFM images of 0.075 mg/mL LDH in the native state (**A**), upon heat treatment (**B**) and upon heat treatment in the presence of 0.075 mg/mL HSP-1/2 (**C**) are shown. Each image is 5 × 5 μM in size.

Distribution density histogram analysis for native LDH (**D**), upon incubation at 48ºC (**E**) and upon incubation at 49 ºC in the presence of HSP-1/2 (**F**).

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. 2.5C). Distribution density histogram shows a size distribution in the range of 2-25 nm for native LDH (Fig. 2.5D) and 5-80 nm for heat treated LDH (Fig. 2.5E). The size distribution shifted towards lower values, viz., 5-35 nm (Fig. 2.5F) 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.

2.4.3. 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. 2.6. ADH showed a rapid increase in the turbidity when incubated at 48ºC, which reached a maximum and remained steady thereafter (Fig. 2.6A, curve 1). Presence of HSP-1/2 (0.01 mg/mL) resulted in a significant reduction in the aggregation (~60% reduction, curve 2). However, pre-incubation of HSP-1/2 with PrC reversed this in a concentration dependent manner (curves 3-5). ADH alone shows ~50% aggregation at ~150 seconds (curve 1), whereas in the presence of HSP-1/2 (0.01 mg/mL), even at 900 seconds the aggregation does not approach 50% of that obtained with ADH alone (curve 2). When HSP-1/2 was pre-incubated with PrC, 50% aggregation was observed at 360, 260 and 165 seconds 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 seconds under different conditions is shown in Fig. 2.6B. 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

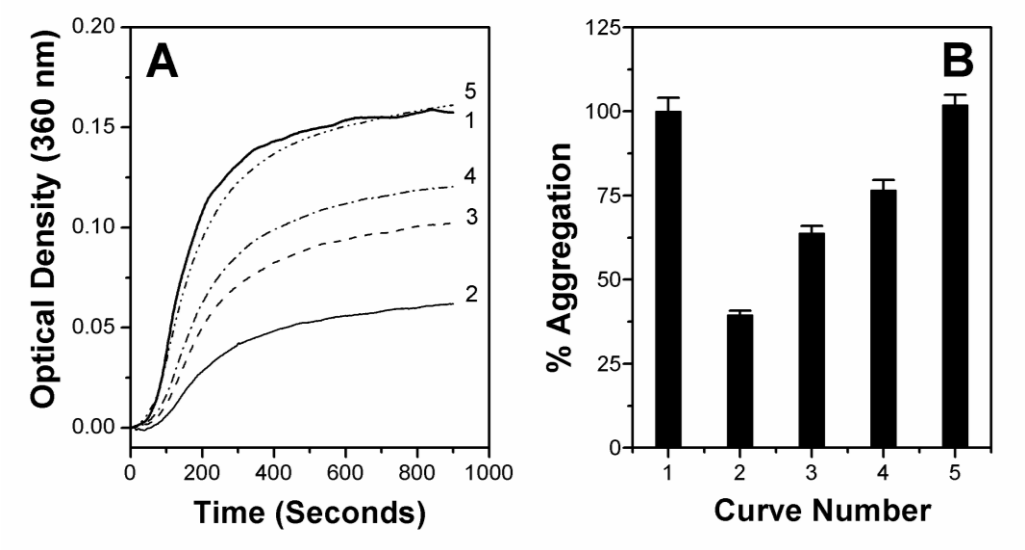


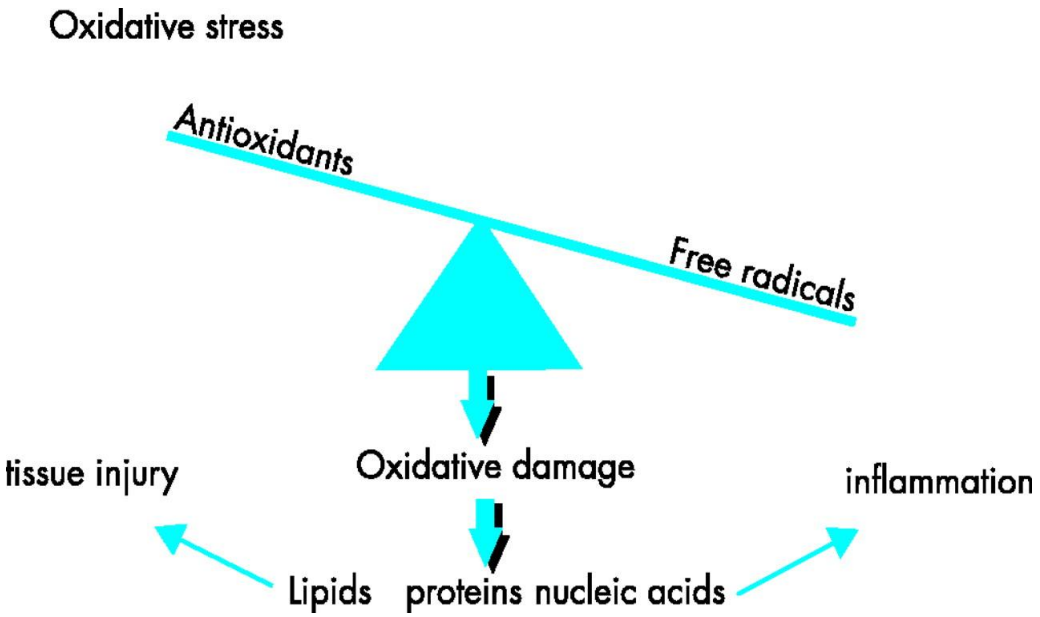
Fig. 2.6. Effect of phosphorylcholine binding on the chaperone-like activity of HSP-1/2. **A**) Aggregation profiles of ADH in the absence and presence of HSP-1/2 and phosphorylcholine. The samples are: (1) ADH at 48°C, (2) ADH + HSP-1/2, (3) ADH + HSP-1/2 + 0.5 mM PrC, (4) ADH + HSP-1/2 + 1 mM PrC and (5) ADH + HSP-1/2 + 2 mM PrC. ADH and HSP-1/2 concentrations were 0.05 mg/mL and 0.01mg/mL, respectively in all the samples. **B**) Bar diagram representing the aggregation of ADH in the various samples

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 [Calvete et al., 1997]. 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 [Sankhala & Swamy, 2010]. 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 [Sankhala & Swamy, 2010]. 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.

Chaperone-like activity of HSP-1/2, under oxidative stress



(Adopted from *Occup. Environ. Med.* (2003) 60: 612-616)

The work presented in this chapter is to be communicated

Chapter 3

3.1. Summary

The major protein of equine seminal plasma, HSP-1/2 exhibits chaperone-like activity (CLA) against a variety of target proteins by protecting them against thermal and chemical stress conditions and redirect them towards active confirmation. In the current study, it is shown that HSP-1/2 can act as a chaperone under oxidative conditions. HSP-1/2 is able to protect enzymes such as ADH, G6PDH against H_2O_2 induced oxidative stress. Further. the present studies show that HSP-1/2 also inhibits lipid (linoleic acid) peroxidation against hydroxyl radicals *in vitro*. These results are of great significance considering limited or no anti-oxidative mechanism has been reported to be present in the mammalian spermatozoa that prevents lipid peroxidation which is detrimental to motility and functioning of spermatozoa.

Chapter 3

3.2. Introduction

The Oxygen paradox is a necessary evil in most of the cellular systems. Cells under aerobic conditions constantly produce and balance the reactive oxidative species (ROS) and antioxidants. ROS production is beneficial as well as detrimental to the cells. The controlled generation and regulation of ROS is required for many cellar functions such as innate immune system, signaling cascade and deactivation of various physiological processes. When the regulation of ROS and antioxidants is imbalanced towards ROS, causing deleterious effects on lipids, proteins and DNA in biological system, it is said to be under "oxidative stress" [Agarwal et al., 2003]. Similar to other cells, spermatozoa also produce ROS through a mechanism similar to that of leukocytes, which involves membrane bound NADPH oxidase [Henkel, 2011; Aitken et al., 1997]. Higher concentration of ROS levels were observed in the impaired/defective sperm cells which are associated with loss of motility, decreased sperm-egg interaction and loss of fertility [de Lamirande and Gagnon, 1992a; Aitken et al., 1989; Blondin et al., 1997]. At lower concentrations, ROS produced by spermatozoa is found to be involved in signaling processes controlling sperm capacitation and sperm-egg interaction [de Lamirande and Gagnon, 1993a, 1993b; Griveau et al., 1994, 1995b]. This balance of ROS production in the semen is maintained by various factors present in the sperm and seminal plasma [Agarwal et al., 2014].

Seminal plasma is a fluid medium which acts as a carrier of spermatozoa from testis to female reproductive tract. Seminal plasma acts as a nutritional medium for sperm cells and also protects spermatozoa from neutrophils in female reproductive tract [Yanagimachi, 1994; Alghamdi et al., 2004; Assreuy et al., 2002; Doty et al., 2011]. Seminal plasma mainly contains enzymes, glutathione peroxidase/reductase, superoxide dismutase and catalase as the ROS scavenging

enzymatic system. Apart from these enzymes, it also contains several small molecules such as ascorbic acid, albumin, carnitine, hypotaurine, tocopherol, taurine and urate, which posses antioxidant activity. The above mentioned small molecules along with enzymes together are responsible for the ROS scavenging capacity of seminal plasma [Balercia et al., 2003; Smith et al., 1996].

Equine seminal plasma constitute >90% of low molecular weight non enzymatic proteins, designated as HSP-1 to HSP-8. Among these, HSP-1 and HSP-2 are the major proteins of equine seminal plasma and account for more than 70% of total protein content [Calvete et al., 1994]. HSP-1 and HSP-2 are present as sperm bound and sperm free (in seminal plasma) forms and are important for cholesterol efflux, a maturation step without which the sperm cannot interact with egg. HSP-1 is a polypeptide containing 121 amino acids with two fibronectin type-II (FnII) domains and an N-terminal flanking region with four glycosylation sites. HSP-2 has the same primary sequence as that of HSP-1 but lacks a 15 amino acid N-terminal segment and is glycosylated to a lesser extent. Their non-seperable mixture is referred as HSP-1/2 [Calvete et al., 1995]. HSP-1/2 binds to a variety of ligands such as choline phospholipids, gelatin, heparin, LDL and α-lactalbumin [Plante et al., 2015]. Among these, HSP-1/2 binding to the choline phospholipids is investigated in detail as the mode of interaction of HSP-1/2 to the sperm plasma membrane is found to be through choline phospholipids [Greube et al., 2004; Calvete et al., 1997; Kumar et al., 2015].

Recently, HSP-1/2 and PDC-109, a bovine analogue of HSP-1/2 are shown to exhibit/possess chaperone-like activity (CLA) by protecting various target proteins and enzymes against thermal and chemical stress conditions [Sankhala and Swamy, 2010; Sankhala et al., 2012]. It is also found that PDC-109 bound to phospholipids membranes shows higher chaperone-like activity due to an increase in the hydrophobicity of the overall system (PDC-109-lipid complex) [Sankhala et al.,

2011]. Loss of polydispersity is shown to inhibit CLA in both cases (HSP-1/2 and PDC-109) indicating that polydispersity is an important factor for the CLA of these proteins. Recent studies have also shown that CLA of HSP-1/2 is inversely correlated with membrane destabilizing activity and this dual functionality is regulated by a pH switch [Kumar and Swamy, 2015]. All these results establish that HSP-1/2 is able to provide protection to other proteins against chemical and thermal stress conditions *in vitro*. In view of day to day experience of spermatozoa and associated proteins of semen for oxidative stress in the reproductive tract, in the present study, we investigated HSP-1/2 CLA under oxidative conditions and antioxidant properties of HSP-1/2.

3.3. Materials and methods

3.3.1. Materials

Alcohol dehydrogenase, aldolase, fluorescein, linoleic acid, phosphoryl choline chloride calcium salt (PrC), 2.2'- diphenylpicrylhydrazyl (DPPH) and heparin-agarose type-I beeds were purchased from Sigma (St. Louis, MO, USA). p-Aminophenyl phosphoryl choline (PPC) column was obtained from Pierce Chemical Co. (Oakville, Ont., Canada). All other chemicals were obtained from local suppliers are of highest purity available.

3.3.2. Purification of HSP-1/2

HSP-1/2 was purified from equine seminal plasma as described in chapter 2. Briefly, seminal plasma was subjected to affinity chromatography on Heparin-agarose and p-aminophenylphosphorylcholine columns followed by RP-HPLC. The purified HSP-1/2 was extensively dialyzed against 50 mM phosphate buffer, pH 7.4 containing 150 mM NaCl (PBS) and stored at 4 °C until further use.

3.3.3. Alcohol dehydrogenase activity under oxidative stress

Alcohol dehydrogenase activity assay under oxidative stress condition was carried out according to Men & Wang [2007] with slight modification. The catalytic activity of ADH is to oxidize alcohol to aldehyde with simultaneous reduction of NAD^+ to NADH. The reaction was initiated by the addition of 400 mM ethanol to the reaction mixture containing yeast ADH (0.5 μg/mL) and 2.5 mM NAD^+ and increase in the absorption at 340 nm due to the formation of NADH was monitored using a Cary 100 UV-Vis spectrophotometer, which was used for all the other spectrophotometric studies as well. To investigate the effect of oxidative stress on the ADH activity, ADH was pre-incubated with 50 mM H_2O_2 for 10 min and the enzyme activity was measured as described above. To investigate the effect of HSP-1/2 on the ADH activity, HSP-1/2 was pre-incubated with ADH for 5 min before the addition of H_2O_2. Relative activities of various treated samples were normalized with respect to the native activity of the enzyme. All experiments were done in triplicates and the average values are reported.

3.3.4. G6PDH assay under oxidative conditions

G6PDH activity was assayed by a spectrophotometric method essentially as described earlier [Sankhala et al., 2012]. G6P is oxidized to 6-phospho-D-gluconate by G6PDH with simultaneous reduction of NADP to NADPH. The reaction was initiated by the addition G6P (5 mM) of to a mixture containing G6PDH (0.5 μM), NADP (0.5 mM) and 12 mM each of $MgCl_2$ and KCl in Tris buffer pH 7.4, and increase in absorbance at 340 nm due to the formation of NADPH, was monitored. To optimize the oxidative conditions, stock solution of 1M H_2O_2 was prepared and different aliquots were added with different incubation time to G6PDH before carrying out the activity assay. To investigate the effect of HSP-1/2 on the enzyme

activity under oxidative stress, 0.5 μM of G6PDH was incubated with 50 μg or 100 μg of HSP-1/2 before incubating with H_2O_2. Relative activities of various treated samples were normalized with respect to the native enzyme activity. All the results reported are averages of at least three independent experiments.

3.3.5. Linoleic acid peroxidation assay

Linoleic acid peroxidation assay was performed spectrophotometrically [Barriere et al., 2001]. A 20 mM stock solution of linoleic acid was prepared in ethanol and ROS was generated from $FeSO_4$ and sodium ascorbate system. A 10 μl alliquot of stock solution was added to give a final conventration of 0.2 mM linoleic acid to a solution containing $FeSO_4$ (100 μM) and different concentrations of HSP-1/2. ROS were generated by addition of ascorbate (10 μM). A control reaction carried out in the absence of HSP-1/2. Formation of conjugated diene hydro peroxides due to oxidation was monitored at 234 nm at 37 °C. The protective activity of HSP-1/2 was defined as [1- (A_{234} sample/ A_{234} control)] X 100. All the results presented are average values from at least two independent experiments.

3.3.6. Hydroxyl radical (·OH) prevention assay using fluorescein

Hydroxyl radical prevention was assayed using fluorescein as a marker [Ou et al., 2002]. Briefly, 50 mM H_2O_2 was added to the reaction mixture contained 0.2 μM fluorescein in PBS. ROS generation was initiated by the addition of 10 μL of Co^{2+} (100 μM). The same reaction was carried out in the presence of various additives as indicated. Fluorescence decay profile of fluorescein was monitored at 515 nm in an ISS PC1 Spetrofluorimeter with the excitation wavelength set at 493 nm. Slit widths of 4 nm were used for both excitation and emission monochromators. The initial fluorescence intensities of fluorescein (after the addition of Co^{2+}) were normalized. The percent inhibition of hydroxyl radical was calculated using the formula (100-

(F_C/F_O) X 100), where F_O and F_C are the normalized fluorescence intensities in the absence and in the presence of various additives at 60 minutes. All the reported results are averages of at least two independent experiments.

3.3.7. DPPH assay

Radical scavenging capacity of HSP-1/2 was probed using DPPH assay according to earlier protocol [Sharma and Bhatt, 2009]. Buffered methanolic solution of DPPH (50 μg) was incubated with different concentrations of HSP-1/2 or buffer alone for 30 min in dark before measuring the absorbance at 517 nm. Ascorbic acid was taken as positive control for the experiment.

3.3.8. Oxidation of HSP-1/2

HSP-1/2 was subjected to different oxidation conditions with respect to incubation time and concentration of oxidants used. In HSP-ox1, HSP-1/2 (0.5 mg/ml) was incubated with 500 μM sodium ascorbate, 100 μM $FeCl_3$ and 2 mM H_2O_2 for 24 hours in PBS. For HSP-ox2, HSP-1/2 (0.5 mg/ml) was incubated with 500 μM sodium ascorbate, 100 μM $FeCl_3$ and 5 mM H_2O_2 for 48 hours in PBS at room temperature. The reaction was stopped by adding 10 μL of catalase (10 U/μL) and after 5 minutes of incubation, the mixture was cooled on ice, dialyzed and stored at 4 °C.

3.3.9. Chaperone-like activity assay

Chaperone-like activity of HSP-1/2 was assessed by monitoring the ability of HSP-1/2 to suppress heat induced aggregation of target proteins such as ADH and ALD as described earlier [Sankhala et al., 2012]. Briefly, ALD (0.1mg/ml) alone or in the presence of different concentrations of native HSP-1/2 and differentially oxidized HSP-1/2 (HSP-ox1, HSP-ox2) were incubated at 48 °C and aggregation of

the sample was monitored at 360 nm using a Cary 100 Uv-Vis spectrophotometer equipped with a Peltier temperature controller. Aggregation profiles of ALD alone was taken as 100% and percent aggregation of other samples was calculated with respect to the native enzyme.

3.3.10. Erythrocyte lysis assay

Erythrocyte lysis assay was performed as described earlier [Damai et al., 2010]. A 100 µL alliquot of 4% human RBC suspension in PBS was mixed with 50 µg of HSP-1/2 or HSP-ox1 or HSP-ox2 and the final volume was adjusted to 0.5 ml with PBS. The Reaction mixture was then incubated for 1 hour before centrifuging samples at 5000 rpm for 5 minutes. Supernatant was collected and its absorbance was measured at 415 nm.

3.4. RESULTS and DISCUSSION

3.4.1. Alcohol dehydrogenase activity under oxidative stress

Alcohol dehydrogenase (ADH) from yeast is a zinc containing tetrameric enzyme with multiple disulphide linkages. Under oxidative stress conditions ADH activity of conversion of alcohol to aldehyde with simultaneous reduction of NAD^+ to NADH is lost due to conversion of Cys^{43} and Cys^{153} to Cys-SO_2H and Cys-SO_3H, respectively and subsequent loss of zinc from the active site [Men and Wang, 2007]. Here the effect of HSP-1/2 on the activity of ADH under oxidative stress was investigated. As shown in Fig. 3.1A, ADH lost ~70% of the activity in the presence of H_2O_2 compared to that of under native conditions. In the presence of 10, 25 and 50 µg HSP-1/2 ~48 %, ~ 74% and >94% activity was observed indicating that HSP-1/2 is able to protect ADH against oxidative stress in a concentration dependent manner. HSP-1/2 presence under native conditions did not alter the activity of ADH. A bar

diagram representing percent activity of ADh under different conditions is shown in Fig 3.1B.

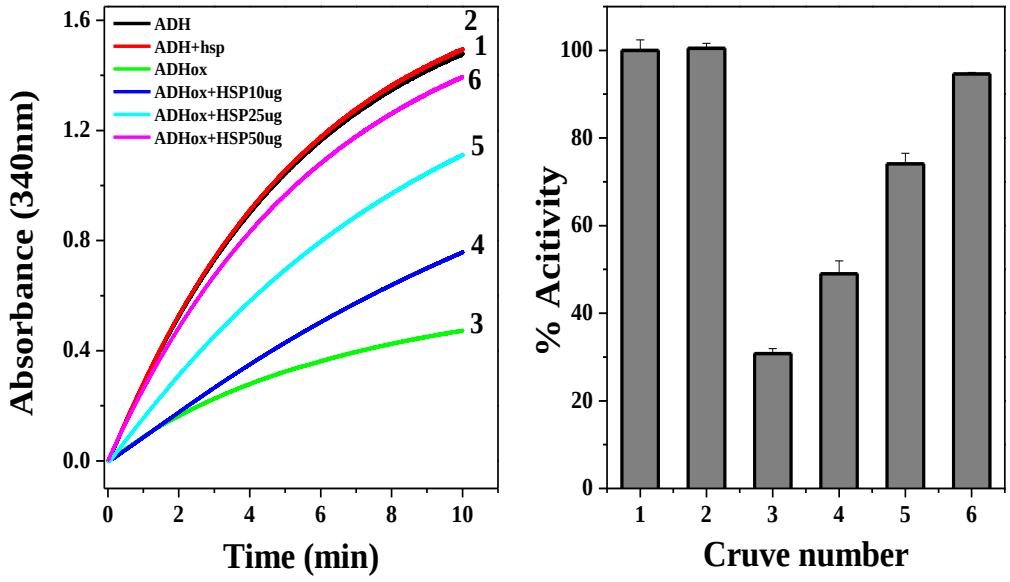


Fig. 3.1. ADH activity assay under oxidative stress conditions. A) Activity of the ADH at room temperature under native conditions (1), after incubation with H_2O_2 for 10 min (2), upon incubation with H_2O_2 in the presence of HSP-1/2 (10 µg) (3), upon incubation with H_2O_2 in the presence of HSP-1/2 (25 µg) (4), upon incubation with H_2O_2 in the presence of HSP-1/2 (50 µg) (5) and after incubation with HSP-1/2 alone (6) are shown. B) Bar diagram representing the activity of ADH at 300 seconds (from panel A).

3.4.2. G6PDH activity under oxidative stress

G6PDH is an important enzyme in pentose phosphate pathway and is involved in production of glutathione which is the only oxidative stress regulating/inhibiting system in erythrocytes. The deficiency of G6PDH leads to a condition called 'hemolytic anemia' [Nkhoma et al., 2009]. G6PDH contains a binding pocket for G6P which is susceptible to oxidation. Radical generating systems such as Fe^{3+}/EDTA, Fe^{3+}/citrate, Fe^{3+}/ascorbate or Fe^{3+}/H_2O_2 lead to decrease in the activity (33). While carrying out the experiments in the presence of Fe^{3+}/H_2O_2

system, we found that H_2O_2 alone was able to decrease the G6PDH activity in a time and concentration dependent manner (Fig 3.2A & B).

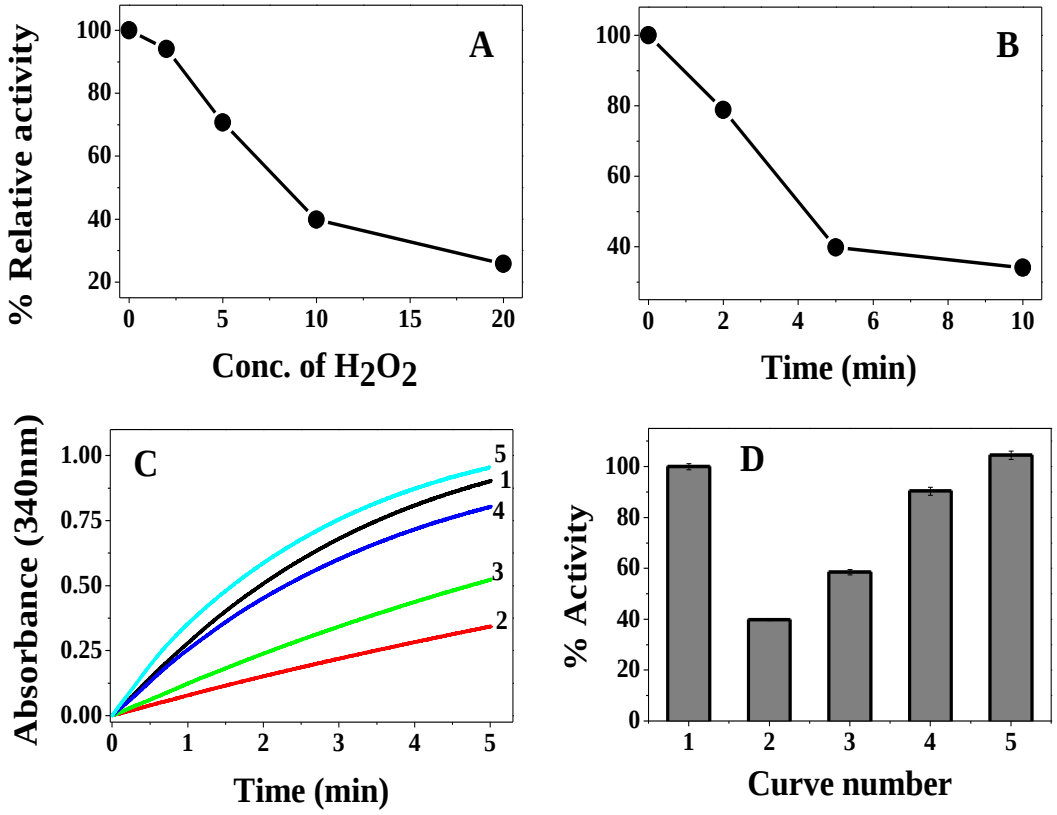


Fig. 3.2. G6PDH activity assay under oxidative stress conditions. A) G6PDH activity in the presence of different concentrations of H_2O_2 when incubated for 5 min. B) Time dependent loss of activity of G6PDH in the presence of 10mM of H_2O_2. C) Activity of the enzyme at room temperature under native conditions (1), after incubation with 10 mM H_2O_2 for 5 min (2), upon incubation H_2O_2 with in the presence of HSP-1/2 (50 µg) (3), upon incubation H_2O_2 with in the presence of HSP-1/2 (100 µg) (4) and after incubation in the presence of HSP-1/2 alone (5). D) Bar diagram representing the activity of G6PD at 300 seconds (from panel C).

Further, effect of HSP-1/2 on the oxidative stress induced decrease in the activity of G6PDH was investigated. G6PDH shows ~40% residual activity under

oxidative stress conditions (Fig 3.2C, curve 2). In the presence of 50 μg of HSP-1/2 ~59% activity is retained and in the presence of 100 μg of HSP-1/2 >90% activity is observed indicating the protecting ability of HSP-1/2 towards oxidative stress induced loss of G6PDH activity. As a control, when the assay was carried out in the presence HSP-1/2 pre-incubated with G6PDH without H_2O_2, more than 4% increase in the activity was observed consistently, similar to earlier observation. This could be because of refolding of any denatured G6PDH in the presence of HSP-1/2 as observed earlier with PDC-109 (Sankhala & Swamy, 2010). It is also note worthy that small heat shock proteins such as Hsp27 is found to increase the G6PDH activity under oxidative conditions [Preville et al., 1999]. The above finding is of significant importance as G6PDH is present in sperm cells and on the sperm plasma membrane and is found to be involved in the metabolic pathway.

3.4.3. Lipid peroxidation

Polyunsaturated fatty acids are present in high concentrations in plasma membranes of most of the mammalian spermatozoa, including equine [Storey,1997]. Their presence is important for acrosome reaction as they provide fluidity and fucogenisity to the sperm plasma membrane. But these fatty acids are highly susceptible to the peroxidation processes initiated by ROS and propagate through chain reaction within the membrane but have very limited repair mechanism. Hence any damages to them results in loss of sperm structural integrity and function as well as decreased sperm-egg interaction [Blondin et al., 1997; Rao et al., 1989]. To investigate the protective effect of HSP-1/2 towards lipid peroxidation, linoleic acid peroxidation assay was employed. Under oxidative stress conditions, a steep increase in the absorption at 234 nm with time was observed, which corresponds to diene hydroperoxides produced from oxidation of linoleic acid. This absorption over a peroid of time levels off (Fig 3.3A). In the presence of HSP-1/2 the absorption is

decreased in a concentration dependent manner indicating decrease in lipid diene peroxide formation. In the presence of 10 µg/mL and 25 µg/mL HSP-1/2 ~25% and 47% respectively, of hydroperoxide production was decreased,. Whereas in the presence of 50 µg/mL of HSP-1/2, more than 75% of decrease in diene hydroperoxides is observed compared to control. These results indicate that HSP-1/2 is able to prevent lipid peroxidation. This is a significant result as HSP-1/2 is found to be present on the sperm plasma membrane, which is the site of lipid peroxidation *in vivo*. These results are also of commercial importance as the sperm storage in the semen extenders are found to result in increased levels of sperm lipid peroxidation which is detrimental to spermatozoa. As HSP-1/2 is found to inhibit the lipid peroxidation, addition of HSP-1/2 in to the semen extenders is recommended [Ball and Vo, 2002].

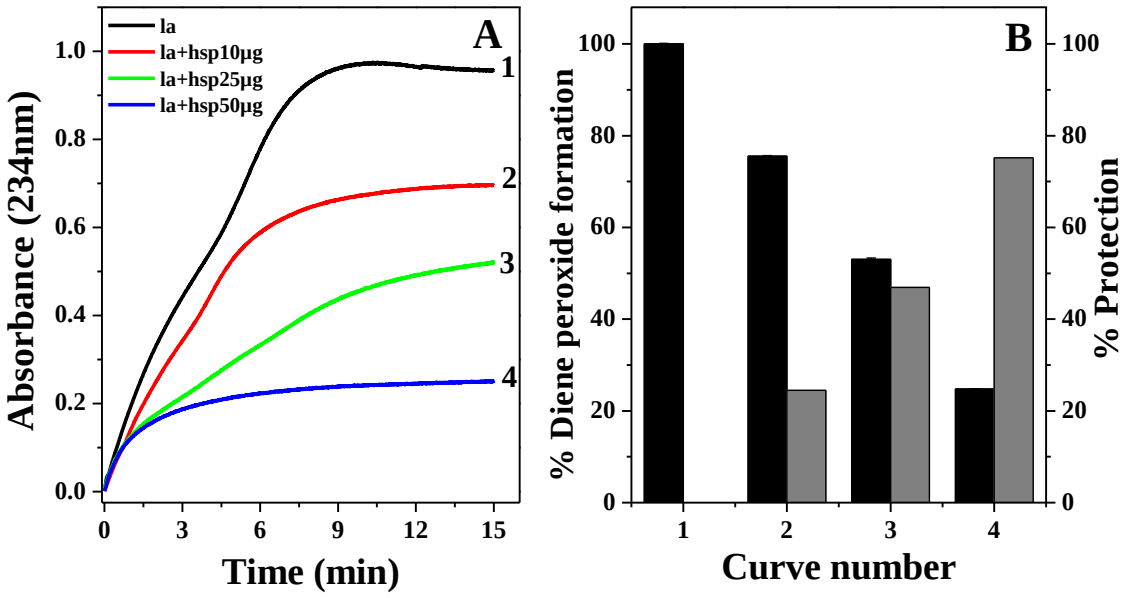


Fig. 3.3. Linoleic acid peroxidation assay. A) Kinetics of linoleic acid peroxidation by Fe^{3+}/ascrobate alone (curve 1) and in the presence of 10 µg (curve 2), 25 µg (curve 3), 50 µg (curve 4) of HSP-1/2. B) Bar diagram showing percent radical production (black bars) and percent protection offered by HSP-1/2 (gray bars) corresponding to the curves in fig A.

3.4.4. Fluorescein assay

Hydroxyl radicals are the side products of the glycolysis and reactions involving H_2O_2. It has been shown that in the sperm related oxidative stress conditions hydroxyl radicals are involved in the damaging mechanisms but not nitroxide or super oxide, as they have high permeability across the cell membrane barrier [Baumber et al., 2001]. It was shown that fluorescein is an excellent probe to monitor the production of hydroxyl radicals *in vitro*. Under aerobic conditions, hydroxyl radicals produced from fenton reaction: in the presence of H_2O_2 + Metals viz. Co (II)/ Fe (II)/ Cu (I).

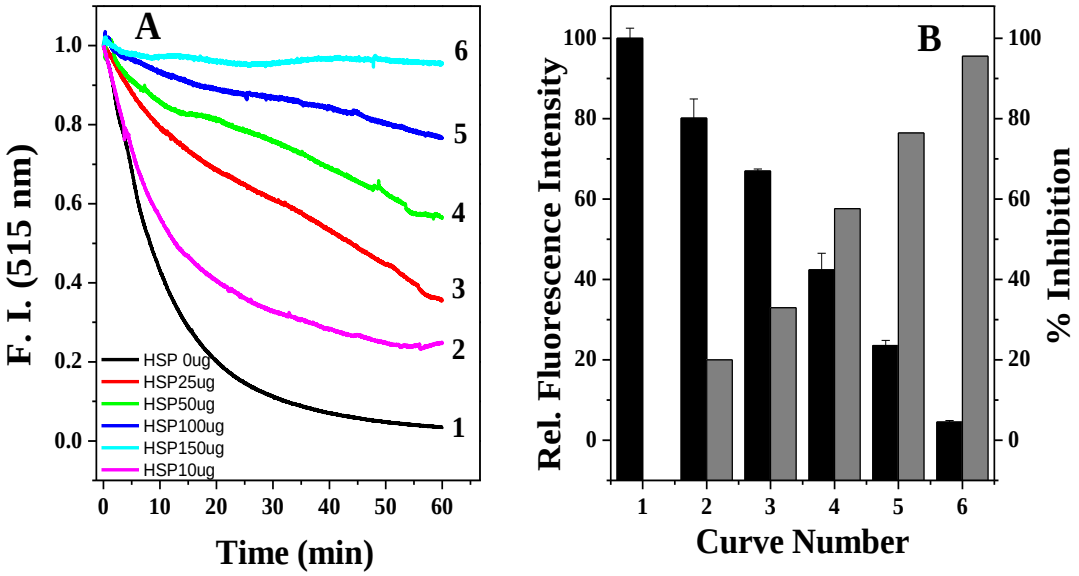


Fig. 3.4. Fluorescein assay for prevention of hydroxyl radical. A) Generation of hydroxyl radicals leading to decrease in dye fluorescence upon incubation with Co^{2+}/ H_2O_2 system in the absence of HSP-1/2 (curve 1), in the presence of HSP-1/2: 10 µg/mL (curve 2), 25 µg/mL (curve 3), 50 µg/mL (curve 4),100 µg/mL (curve 5) and 150 µg/mL (curve 6). B) Bar diagram representing relative fluorescence intensity (black bars) and percent ROS inhibition (grey bars) by HSP-1/2.

Here, we employed a protocol developed earlier by Ou et al. [2002] to investigate the effect of HSP-1/2 on the hydroxyl radical generation/prevention (Fig 3.4A). In presence of hydroxyl radicals fluorescein (FL) gets oxidized and loses its fluorescence as shown in curve 1. In the presence of HSP-1/2 the loss of fluorescence is inhibited in a concentration dependent manner. In the presence of 25 μg and 50 μg HSP-1/2, more than 33% and 57% of the initial fluorescence is retained. Whereas in the presence of 100 μg of HSP-1/2 ~76% fluorescence is observed and in the presence of 150 μg of HSP-1/2, >95% of fluorescence is observed indicating only 4-5% loss in fluorescence intensity compared to the blank (fluorescein alone). A bar diagram showing percentage loss of fluorescence intensity corresponding to different conditions is shown in Fig 3.4B. Hydroxyl radical prevention of gallic acid is taken as positive control and FL without H_2O_2 is taken as negative control. To show the HSP-1/2 specificity, aldolase was taken as negative control, which does not show any protection towards loss of fluorescence of FL. These results indicate that HSP-1/2 can either prevent hydroxyl radical generation, thereby protecting FL from free radicals or scavenge the radicals generated.

3.4.5. Radical scavenging capacity of HSP-1/2

To investigate details of the mechanism of protection offered by HSP-1/2 towards ROS, DPPH radical scavenging assay was performed. DPPH is a stable free radical and in the presence of hydrogen donors, the radical gets paired resulting in decrease in absorbance at 517 nm. DPPH alone (50 μg) has showed an absorbance of ~0.58 and in the presence of 50 and 100 μg of HSP-1/2, no decrease in the absorbance was observed (Fig 3.5), indicating that HSP-1/2 does not act as a hydrogen donor, unlike many small antioxidant molecules such as ascorbic acid or polyphenols.

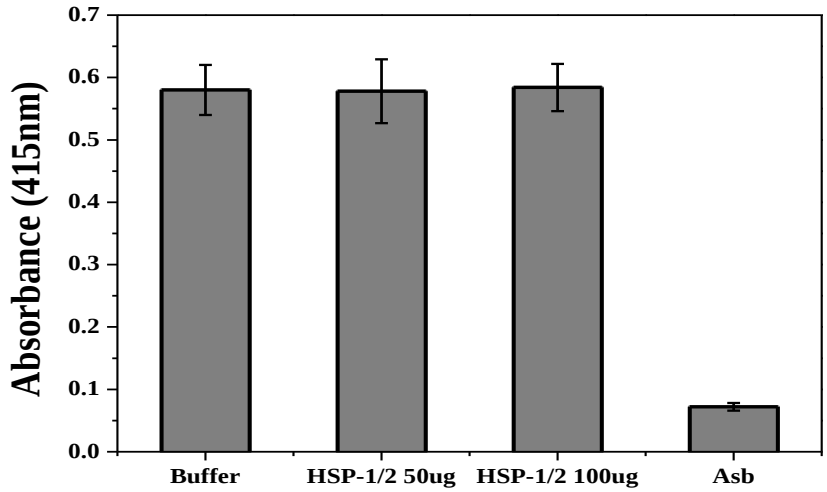


Fig. 3.5. DPPH radical scavenging assay. Absorbance corresponding to DPPH radical alone and in the presence of HSP-1/2 (50, 100 µg) and ascorbic acid (10 µM).

3.5.5. Effect of oxidation on chaperone like activity of HSP-1/2

α-Crystallin, a well known heat shock protein which exhibits chaperone-like activity against various target proteins under oxidative, thermal and chemical stress is found to be susceptible to oxidation [Wang and Spector, 1995]. Oxidation of α-crystallin leads to oxidation of the thiol groups, which was shown to have moderate effect on the chaperone-like activity of the protein. This prompted us to verify whether oxidation of HSP-1/2 itself has any effect on the functional properties of HSP-1/2 (Fig 3.6).

3.5.5.1. Effect of oxidation on inhibition of heat induced aggregation of target proteins by HSP-1/2

HSP-1/2 shows protective activity towards heat induced inactivation of G6PD and protects various other enzymes such as ADH, ALD, CA and LDH against heat induced aggregation [Sankhala et al., 2012]. Aggregation assays were employed to investigate the effect of oxidation on the ability of HSP-1/2 to protect the target

proteins under thermal stress. As shown in the Fig. 3.6 HSP-1/2 under normal conditions inhibits heat induced aggregation of aldolase (Fig 3.6A &B). In the presence of 15 μg of HSP-1/2, aldolase shows ~60% of aggregation compared to aldolase alone. In the presence of oxidized HSP-1/2, HSP-ox1 and HSP-ox2, the aggregation increased to 61% and 64% respectively. 25 μg of HSP-1/2 was able to inhibit the aggregation by ~85% showing only 15% aggregation compared to aldolase alone. No change in protective ability was observed In the presence of same concentration of HSP-ox1, whereas a 5% decrease in the protective ability was observed in the presence of HSP-ox2. These results indicate that oxidation of HSP-1/2 has does not have any predominant effect in its ability to protect ALD against thermal stress and that even under oxidative conditions HSP-1/2 exhibits chaperone like activity (CLA).

3.5.5.2. Effect of oxidation on antioxidant property of HSP-1/2 towards lipid peroxidation

To investigate whether oxidation has any effect on the protective property of HSP-1/2 towards lipid peroxidation, linoleic acid assay was employed as described above (Fig 3.6C). In the presence of 50 μg native HSP-1/2, linoleic acid peroxidation under oxidative stress conditions was decreased to ~25%. In the presence of same concentrations of HSP-ox1 and HSP-ox2, ~26.5 and 28.5% of lipid peroxidation was observed, showing a marginal increase of 1.5 and 3.5% respectively, increase in linoleic acid peroxidation. These results indicate that oxidation of HSP-1/2 under the given conditions do not affect its antioxidative properties and even oxidized HSP-1/2 retains inhibition ability towards lipid peroxidation.

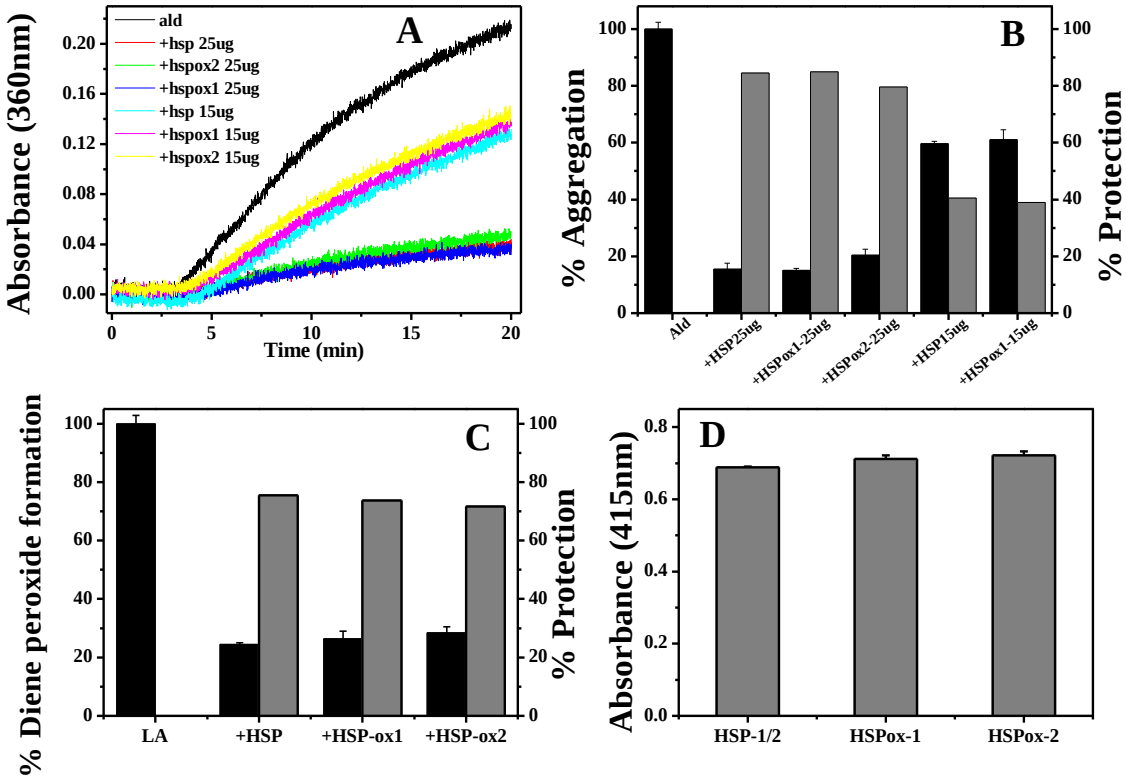


Fig. 3.6. Effect of oxidation on CLA and RBC destabilizing activites of HSP-1/2. A) Aggregation profiles of aldolase (0.1mg/mL) alone and in the presence of native HSP-1/2, HSP-ox1 and HSP-ox2. B) Bar diagram showing percent aggregation of aldolase (black bars) and percent protection (gray bars) offered by HSP-1/2. C) Bar diagram showing percent diene peroxide formation with linoleic acid alone and in the presence of HSP-1/2 (native and oxidized) and percent protection offered by HSP-1/2. D) Erythrocyte lysis carried out in the presence of 50 µg each of native HSP-1/2, HSP-ox1 and HSP-ox2.

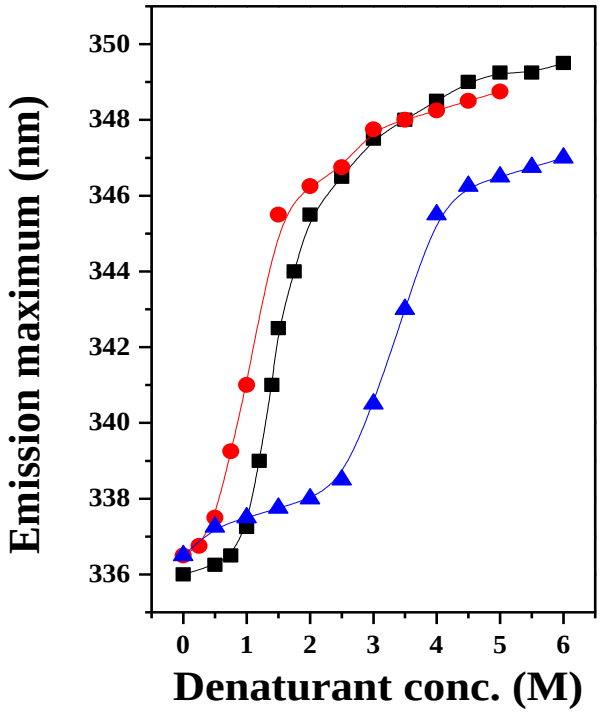
3.5.5.3. Effect of oxidation on erythrocyte membrane destabilizing effect of HSP-1/2

HSP-1/2 is shown to posses destabilizing activity towards model cell membranes such as erythrocytes. Erythrocytes incubated in the presence of HSP-ox1 and HSP-ox2 showed similar lysis to that of native HSP-1/2. Only 3% and 5% increase in the RBC lysis was observed in the presence of HSP-ox1 and HSP-ox2,

respectively compared to native HSP-1/2 (Fig 3.6D). This marginal increase can be due the presence of trace amounts of Fe^{+3}/ascorbate in the system. These results indicate that oxidation of HSP-1/2 does not have any effect on its destabilizing property towards erythrocytes. All the above experiments indicate that even under oxidized conditions HSP-1/2 can exhibit its chaperone-like and membranolytic activities.

In summary, we presented several lines of evidence, which show that HSP-1/2 can protect enzymes such as G6PDH, ADH and inhibits linoleic acid peroxidation against ROS induced oxidative stress. These results are of particular interest/importance because of very few anti-oxidative systems present in the seminal plasma and spermatozoa. As described earlier, protection of target proteins by HSP-1/2 against heat and chemical stress is of important considering their sub-physiological conditions whereas oxidative stress condition is a day to day customary stress that spermatozoa experience in their production, growth, survival and function [Agarwal et al., 2003; Henkel, 2011]. Hence, chaperone like activity of HSP-1/2 against oxidative stress condition can be of great relevance considering their high concentrations in the seminal plasma and their presence in other mammalian reproductive systems.

Fluorescence investigations on choline phospholipid binding and chemical unfolding of HSP-1/2



Chaotrope induced unfolding of HSP-1/2 in the presence of DTT

The work presented in this chapter is communicated

Chapter 4

4.1. Summary

In the present study, the heterogeneity and microenvironment of tryptophan residues in HSP-1/2 was investigated in the native state, in presence of PrC and phosphatidylcholines with short (valeroyl, C-5) and long (myristoyl, C-14) chains, and upon denaturation using fluorescence quenching, time-resolved fluorescence and red-edge excitation shift (REES) measurements. The results obtained show that the environment of tryptophan residues in HSP-1/2 is more heterogeneous as compared to that in PDC-109. Binding of choline phospholipids afforded a protection to the tryptophan residues with the shielding order being: PrC ≤ divalaroyl PC < dimyristoyl PC. REES value obtained for HSP-1/2 (3.5 nm) is smaller than that of observed for PDC-109 (4 nm) and binding to PrC and DVPC reduced the REES to 1 nm. This observation indicates short chain phospholipids don't have much change effect compared to PrC. HSP-1/2 exhibits only partial unfolding with chemical denaturants with no cooperativity, whereas complete unfolding was observed in the presence of 10 mM dithiothreitol, indicating that disulphide linkages prevent complete unfolding of the protein. In the presence of PrC the transition midpoints shifted to higher concentrations of the denaturant together with a broadening of the sigmoidal transitions, indicating that ligand binding as well as polydispersity modulate the unfolding process.

Chapter 4

4.2. Introduction

As mentioned in chapter 1, HSP-1 and HSP-2, the major proteins of equine seminal plasma, belong to the super family of fibronectin type-II (FnII) proteins. PDC-109, which is the bovine homologue of HSP-1/2, is the most well characterized protein of this family [Fan et al., 2006; Swamy, 2004]. A highly important feature of this family of proteins is that all of them exhibit high affinity for phospholipids containing the choline head group with high affinity and thus bind to the sperm plasma membrane, which is rich in phosphatidylcholine and sphingomyelin [Parks and Lynch, 1992]. Recent work from our laboratory has shown that both PDC-109 and HSP-1/2 exhibit chaperone-like activity [Sankhala and Swamy, 2010; Sankhala et al., 2012], indicating that they may help other proteins in the seminal plasma to achieve the correct folded structure or protect them against various kinds of stress.

While the interaction between PDC-109 and phospholipids has been investigated in significant detail, the information available on the HSP-1/2-phospholipid interaction is rather limited. In one study, using fluorescence and EPR spectroscopy, it was shown that similar to PDC-109, HSP-1/2 also specifically recognizes phospholipids containing the choline head group such as phosphatidylcholine and sphingomyelin [Greube et al., 2004]. The interaction of HSP-1/2 with lipids perturbs the membrane structure by intercalating into its hydrophobic core which results in a rigidification of the membrane. Even though the interaction of HSP-1/2 with phospholipids observed is quite similar to that of PDC-109, quantitative differences are observed in the extent of immobilization of lipid membrane and extent of removal of phospholipids from erythrocyte membrane. Single crystal X-ray diffraction studies on PDC-109/PrC complex have shown that tryptophan and tyrosine residues are involved in a cation-π interaction with the quarternery ammonium group of choline moiety and hydrogen bonding with

phosphate group, respectively [Wah et al., 2002]. Our previous intrinsic fluorescence quenching studies on PDC-109 indicated that binding of the protein to DMPC vesicles most likely results in the insertion of a segment containing Trp-90 into the hydrophobic core of the protein [Anbazhagan et al., 2008], whereas PDC-109/B (domain B) does not interact to the same extent with lipid membranes to that of whole PDC-109 [Damai et al., 2009]. These results point to the importance of aromatic amino acids, especially tryptophan, in the PDC-109/phospholipid interaction and hence in sperm capacitation. Such direct evidence for the involvement of specific amino acid residues in the interaction of HSP-1/2 with lipids is currently not available.

In the present study, different fluorescence approaches have been employed to investigate the conformational changes observed through changes in the intrinsic fluorescence of HSP-1/2 alone and in the presence of different soluble phospholipids. The accessibility of tryptophan residues in HSP-1/2 was investigated by quenching studies employing a neutral quencher, acrylamide and two ionic quenchers, iodide ion and cesium ion with native HSP-1/2 and in the presence of different lipids. In addition, chemical unfolding of HSP-1/2 mediated by chaotropic agents, urea, guanidine hydrochloride and guanidine thiocyanate has been investigated by monitoring the fluorescence emission characteristics of the protein.

4.3. Materials and methods

4.3.1. Materials

Acrylamide, potassium iodide, cesium chloride, guanidinium chloride (Gdm.Cl), guanidinium thiocyanate (Gdm.SCN), urea, dithiotheretiol (DTT), phosphorylcholine chloride calcium salt (PrC), lyso-phosphatidylcholine (Lyso-PC) and heparin-agarose type-I beads were purchased from Sigma (St. Louis, MO, USA).

Dimyristoylphosphatidylcholine (DMPC), divalaroylphosphatidylcholine (DVPC) and egg sphingomyelin (SM) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). *p*-Aminophenyl phosphorylcholine column was obtained from Pierce Chemical Co. (Oakville, Ontario, Canada). All other chemicals were obtained from local suppliers and were of the highest purity available.

4.3.2. Purification of HSP-1/2

HSP-1/2 was purified as described in Chapter 2 from seminal plasma collected from healthy horses by affinity chromatography on heparin-agarose and *p*-aminophenyl phosphorylcholine followed by reverse phase HPLC (RP-HPLC). The purified HSP-1/2 was extensively dialyzed against 50 mM tris buffer, pH 7.4, containing 0.15 M NaCl and 5 mM EDTA (TBS). Concentration of HSP-1/2 was estimated from its extinction coefficient of 2.45 for 1 mg/ml using Bradford method [Bradford, 1976].

4.3.3. Preparation of liposomes

Lipids taken in a glass tube were dissolved in either dichloromethane or dichloromethane-methanol mixture and dried under a gentle stream of nitrogen gas whcih yielded a thin lipid film on the inner walls of the tube. After removing the traces of solvent by vacuum desiccation for 3-4 h, the lipid film was hydrated with TBS to give the desired lipid stock concentration. Small unilamellar vesicles (SUVs) were prepared by homogenizing the lipid mixture with 3-4 freeze-thaw cycles followed by sonication of the lipid suspension for 30 min in a bath sonicator at room temperature.

4.3.4. Steady-state fluorescence measurements

Steady-state fluorescence experiments were carried out on a Spex Fluoromax-3 fluorescence spectrometer at room temperature with excitation and emission band

pass filters set at 3 and 5 nm, respectively. Protein samples ($A_{280} \leq 0.1$) were excited at either 280 nm (for ligand binding studies) or 295 nm (for quenching studies) and the emission spectra were recorded in the 310-400 nm range. Intrinsic fluorescence quenching experiments were carried out by adding small aliquots of the quenchers from 5 M stock solutions in TBS to the protein in the same buffer. Sodium thiosulphate (0.2 mM) was added to the stock solution of KI to prevent the formation of triiodide. All the spectra were corrected for inner filter effect as described in [Lakowicz, 1999]. For the red edge excitation shift (REES) experiments excitation wavelength was varied between 280 and 307 nm and emission spectra were recorded between 315 and 400 nm. The results given are the average of at least two independent experiments (with standard deviations of <5% in all cases).

Equilibrium unfolding experiments were performed by mixing a fixed amount of HSP-1/2 (~3 μM) with increasing amounts of denaturants from stock solutions of 8 M Gdm.Cl, 6 M Gdm.SCN and 10 M urea and incubated overnight. Samples were excited at 280 nm and emission spectra were recorded in the range of 310 to 400 nm.

4.3.5. Fluorescence life time measurements

Time resolved fluorescence measurements were performed using an IBH 5000F (Horiba Jobin Yvon, Edison, NJ) time correlated single photon counting (TCSPC) spertrofluorometer. A pulsed LED (NanoLED-01) with an excitation source of 295 nm with a pulse duration of < 1.0 ns was used. The LED profile corresponding to the instrument response function was measured at the excitation wavelength using Ludox (colloidal silica) as the scatterer. Samples of $OD_{295\ nm}$ <0.07 (≤ 5.0 μM) taken in a quartz cell (10 mm path length) were excited at 295 nm and emission decay profile was monitored at 338 nm for HSP-1/2, 347 nm for denatured

protein, 332 nm for HSP-1/2+ PrC and HSP-1/2 + DVPC, 330 nm for HSP-1/2 in presence DMPC vesicles and lyso-PC micelles, which correspond to wavelengths of maximum fluorescence. All the experiments were performed using emission slits with band pass of >4 nm. To optimize the signal-to-noise ratio, 10000 photon counts were collected in the peak channel. The sample and the scatterer were alternated for every 10% acquisition to ensure compensation for timing and shape drifts occurring during the period of collection. The fluorescence intensity decay curves obtained were deconvoluted with the instrument response function and analyzed using DAS software (Horiba Jobin Yvon, Edison, NJ).

4.4. Results

4.4.1. Effect of ligand binding on the fluorescence emission characteristics of HSP-1/2

Fluorescence spectra of HSP-1/2 alone and in the presence of phosphorylcholine (PrC) and various phospholipids containing choline head group viz., DMPC, Lyso-PC, DVPC as well as DMPG and DMPE are shown in Fig. 4.1 and the changes in the emission characteristics viz., shifts in the emission maximum and emission intensity are presented in Table 4.1. From Figure 4.1 and Table 4.1 it can be clearly seen that the emission maximum HSP-1/2 is at 338 nm, which is blue shifted to 331.5 nm in presence of 20 mM PrC, along with a 14% increase in the emission intensity. In the presence of 1 mM DVPC the emission maximum is seen at 331 nm with a 30% increase in fluorescence intensity, whereas in presence of 0.15 mM Lyso-PC micelles and 0.15 mM DMPC vesicles the emission maximum is further blue shifted to 329.5 and 329 nm with about 46% and 67% increase in emission intensity, respectively.

Table 4.1. Values of fluorescence emission λ_{max} and percent change in emission intensity of PDC-109 and HSP-1/2 in the presence of different ligands/conditions.

Condition	PDC-109		HSP-1/2	
	λ_{max}(nm)	% change	λ_{max}(nm)	% change
Native protein	340.0	----	338.0	----
+ 20 mM PrC	335.5	27	331.5	14
+1 mM DVPC	---	---	331.0	30
+ 0.15 mM Lyso-PC	333.5	54	330.0	46
+ 0.15 mM DMPC	333.0	62	329.5	67
+0.15 mM SM	---	---	330.0	19
+ 0.15 mM DMPG	338.0	18.5	336.5	16
+ 0.15 mM DMPE	340.0	----	335.5	7
+ 6 M Gdm.Cl	348.0	40	347.0	103
+ 6 M Gdm.Cl + 10 mM βME	349.5	40	348.5	65
+ Gdm.SCN			347.5	185
+ 6 M Gdm.SCN + 10 mM βME			349.0	132
+ Urea			341.5	91
+ 6 M Urea + 10 mM βME			347.0	-60

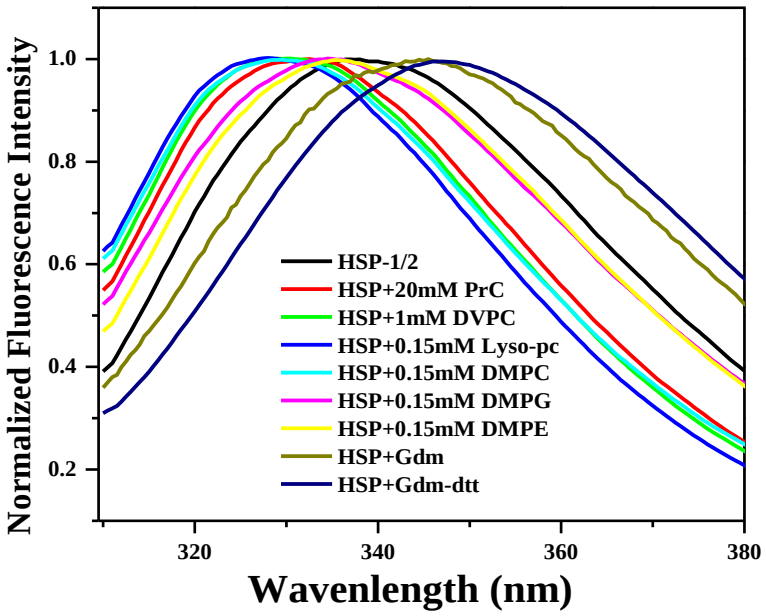


Fig. 4.1. Intrinsic fluorescence emission spectra of HSP-1/2 obtained under different conditions. The samples investigated are indicated in the figure.

Interestingly, despite the presence of a choline head group binding to SM vesicles led to only 19% increase in the emission intensity although the emission λ_{max} exhibited an 8 nm blue shift. Denaturing HSP-1/2 with 6 M Gdm.Cl resulted in a red shift of λ_{max} to 346 nm with about 103% increase in emission intensity. Denaturation with 6 M Gdm.Cl and reducing the disulphide bonds with 10mM DTT led to a further red shift of the emission λ_{max} to 347.5 but only with 65% increase in emission intensity. Emission maxima corresponding to HSP-1/2 under different conditions are given in Table 4.1.

4.4.2. Fluorescence quenching studies of HSP-1/2

Fluorescence spectra corresponding to quenching of HSP-1/2 by acrylamide in the native state and in the presence of different ligands are shown in Figure 4.2.

Quenching profiles of native HSP-1/2 is shown in Fig 4.2A and the quenching profiles of HSP-1/2 complexed with PrC, DVPV and DPMC are shown in Figures 4.2B, 4.2C and 4.2D, respectively. In each case the spectrum exhibiting the highest intensity corresponds to HSP-1/2 in the absence of any quencher, and the spectrum with the lowest intensity corresponds to that recorded in the presence of 0.5M quencher. 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. As shown in Fig 4.2A, acrylamide quenches the fluorescence of the native protein significantly, whereas binding to the above mentioned ligands led to a marked reduction in the extent of quenching. Among the three quenchers employed, acrylamide was the most efficient and quenched the intrinsic fluorescence of HSP-1/2 by ~81% at a final quencher concentration of 0.5 M.

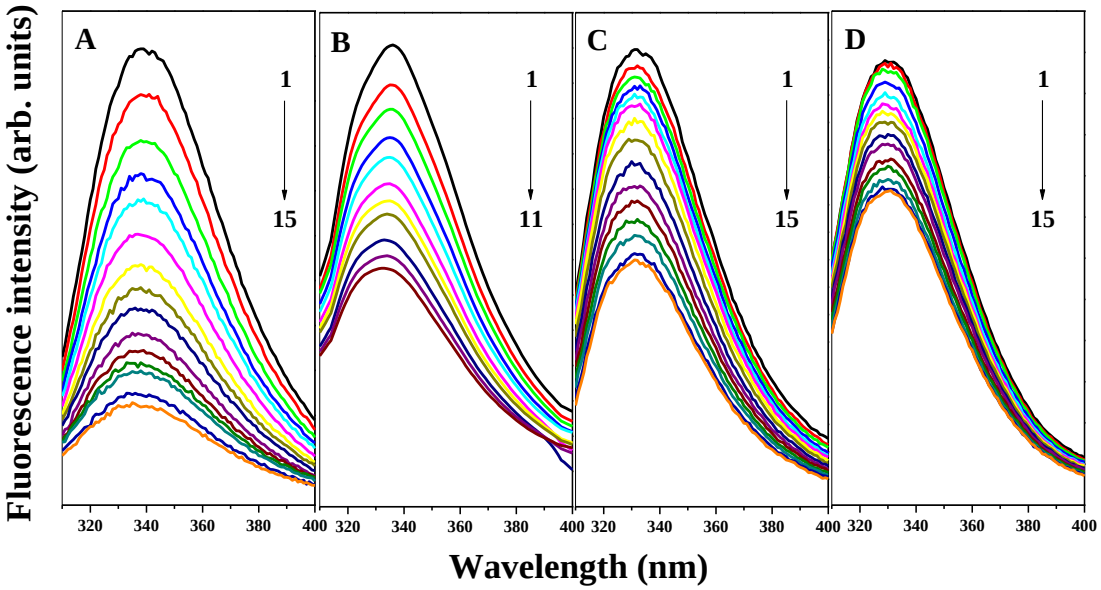


Fig. 4.2. Quenching of HSP-1/2 fluorescence by acrylamide in the native state and upon binding to different ligands. (A) Native HSP-1/2, (B) HSP-1/2 + 20 mM PrC, (C) HSP-1/2 + 1 mM DVPC, (D) HSP-1/2 + 0.15 mM DMPC.

Table 4.2: Parameters obtained from steady state fluorescence quenching of HSP-1/2

Sample description	% Quenching	K_{SV1} (M^{-1})	K_{SV2} (M^{-1})	f_a (%)	K_a (M^{-1})
Acrylamide					
Native HSP-1/2	88.1 (86.2)	8.47	-	91.1	9.97
+ 20 mM PrC	55.4 (65.6)	2.15	3.9	68.1	9.34
+ 1.0 mM DVPC	54.2 (-----)	2.03	-	50.5	8.06
+ 0.15 mM DMPC	28.8 (42.6)	0.81	-	44.2	1.64
Iodide					
Native HSP-1/2	39.1 (35.2)	3.5	0.83	46.0	8.1
+ 20 mM PrC	29.0 (32.7)	1.7	0.61	31.8	7.1
+ 1.0 mM DVPC	28.1 (-----)	1.36	0.43	37.5	6.0
+ 0.15 mM DMPC	15.6 (22.3)	0.79	0.21	20.8	5.4
Cesium ion					
Native HSP-1/2	36.7 (44.8)	0.99	1.64	60.6	2.52
+ 20 mM PrC	17.8 (32.7)	0.34	-	23.6	3.15
+ 1.0 mM DVPC	14.9 (-----)	0.82	0.17	--	--
+ 0.15 mM DMPC	8.9 (15.6)	0.177	-	16.3	1.56

With the ionic quenchers, Cs^+ and I^- 36.5% and 39% quenching was observed, respectively at the same quencher concentration. Similar to PDC-109, the maximum decrease in quenching was observed when the protein was bound to DMPC vesicles. For acrylamide the quenching decreased to 55.4%, 54.1% and 28.8% in presence of 20 mM PrC, 1 mM DVPC and 0.15 mM DMPC, respectively, whereas binding to

DMPC vesicles decreased the quenching by I^- and Cs^+ from 36.5% and 39% to 8.9% and 15.6%, respectively. The extent of quenching observed with different quenchers under different conditions is given in Table 4.2. For comparison, values corresponding to PDC-109 [Anbazhagan et al., 2008] are given in parentheses.

4.4.3. Stern-Volmer analysis of quenching data

The data obtained for the quenching of HSP-1/2 with different quenchers under different conditions were analyzed by the Stern-Volmer equation (Eq.1) and modified Stern-Volmer equation (Eq.2) given below [Lakowicz,1999]:

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

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

where F_o and F_c are fluorescence intensities of the protein recorded in the absence and presence of quencher, respectively. [Q] is the resultant quencher 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 plots for the quenching of HSP-1/2 alone and in the presence of 20 mM PrC, 1 mM DVPC and 0.15 mM DMPC vesicles with acrylamide, iodide and cesium ion are shown in Fig. 4.3A, 4.3B and 4.3C, respectively. Each panel shows the Stern-Volmer plots obtained with HSP-1/2 alone and in the presence of all the three ligands. It can be seen from the Fig. 4.3A that the Stern-Volmer plot for acrylamide quenching of the native protein is linear. However, upon binding to PrC the plot became biphasic with two linear parts having different slopes, whereas the Stern-Volmer plots for quenching in the presence of DVPC and DMPC vesicles are again linear. Stern-Volmer plots obtained for both Cs^+ and iodide ion under native

condition show biphasic patterns. Stern-Volmer plots for quenching by iodide ion in the presence of all the three ligands remain biphasic whereas for quenching by Cs^+ in presence of PrC and DMPC vesicles they become linear. The biphasic pattern indicates heterogeneity in the environment of Trp residues which are accessible to the quencher.

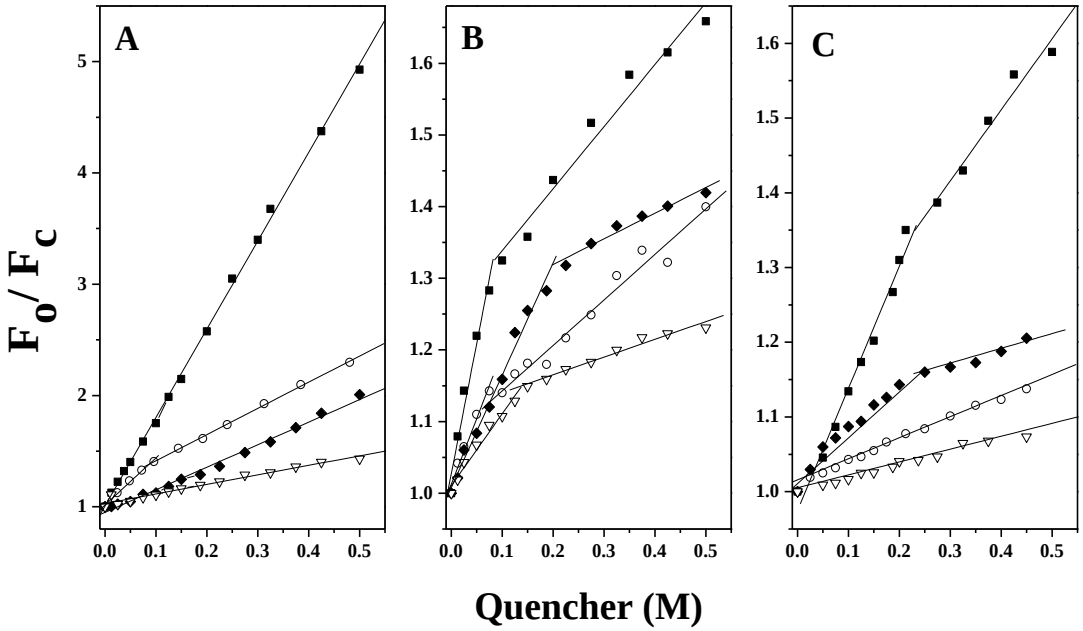


Fig. 4.3. Stern-Volmer plots of fluorescence quenching data for HSP-1/2 obtained for different quenchers. A) Acrylamide, B) Iodide and C) Cesium ion under different conditions: (■) Native HSP-1/2, (○) in presence of 20 mM PrC, (◆) in presence of 1 mM DVPC and (▽) in presence of 0.15 mM DMPC.

Form the slopes of the two linear components two individual Stern-Volmer constants, K_{SV1} and K_{SV2} were determined and are listed in Table 4.2. The Y-intercept of the modified Stern-Volmer plots (Fig. 4.4) for the quenching gives the fraction of accessible fluorophores (f_a) to the quenchers. It can be seen from the data presented in Table 4.2 that more than 90% of the Trp residues are accessible to the neutral quenchers which has only hydrophobic constraint for the penetration in to the

protein matrix. In case of ionic quenchers, I^- and Cs^+, the accessible fraction of fluorophores, f_a decreased to 46% and 60%, respectively. Binding of all the three ligands namely PrC, DVPV and DMPC decreased the accessibility considerably. With all the quenchers employed, HSP-1/2 bound to DMPC has shown least f_a values indicating that DMPC is able to shield the Trp residues to a higher extent from quenchers compared to other ligands.

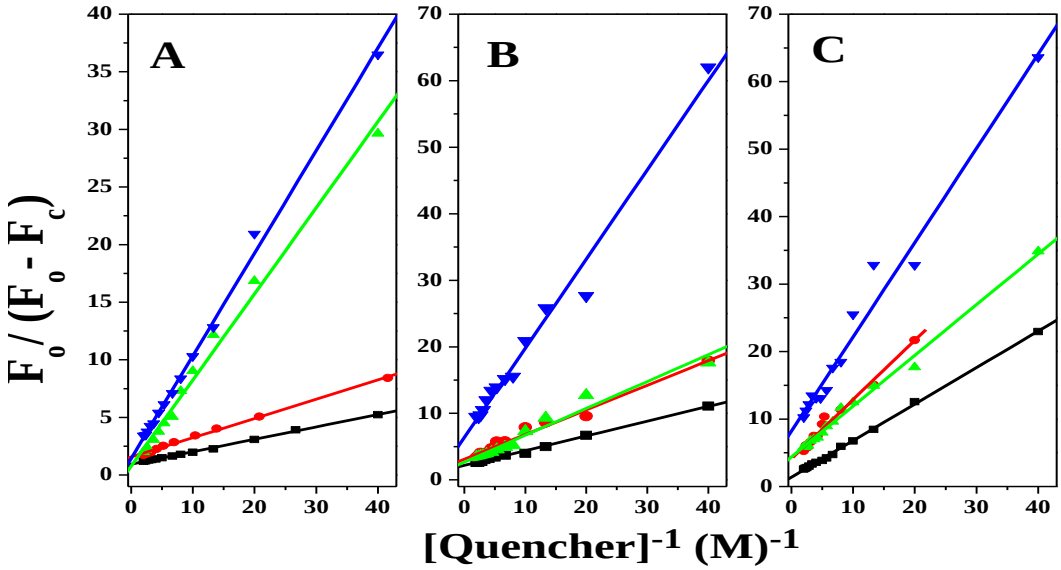


Fig. 4.4. Modified Stern-Volmer plots of fluorescence quenching data for HSP-1/2 obtained for different quenchers. (A) Acrylamide, (B) iodide ion and (C) cesium ion under different conditions: (blue) Native HSP-1/2, (green) in presence of PrC, (red) in presence of DVPC and (black) in presence of DMPC.

4.4.4. Fluorescence lifetime measurements

Fluorescence decay curves of HSP-1/2 in the native state and in presence of DMPC and upon denaturation with 6 M Gdn.HCl are given in Fig. 4.5. In addition, lifetime measurements were also carried out in the presence of various ligands as well as acrylamide. In all the cases the decay curve could be best fitted to a triexponential. Monoexponential and biexponential fittings gave considerably larger

χ^2 values. From the triexponential fits the three decay times $\tau 1$, $\tau 2$ and $\tau 3$ were obtained as 0.39, 1.36 and 5.1 ns, respectively with corresponding weighting factors of 0.56, 0.30 and 0.13. Values of decay times and their corresponding weighting factors, which indicate their relative contribution for all the samples are given in Table 4.3.

The average lifetime of fluorescence decay for HSP-1/2 under different conditions was calculated from life time values τ_i and their corresponding pre-exponential weighing factors, α_i using following equations [Lakowicz, 1999]:

$$\tau = \Sigma_i \alpha_i \tau_i / \Sigma_i \alpha_i \quad (3)$$

$$\langle \tau \rangle = \Sigma_i \alpha_i \tau^2_i / \Sigma_i \alpha_i \tau_i \quad (4)$$

where τ and $\langle \tau \rangle$ are the amplitude and intensity average fluorescence lifetimes, respectively. For native HSP-1/2, τ and $\langle \tau \rangle$ values obtained are 1.32 and 3.14 ns, respectively. These values along with the average life time values obtained for HSP-1/2 under different conditions are also reported in Table 4.3.

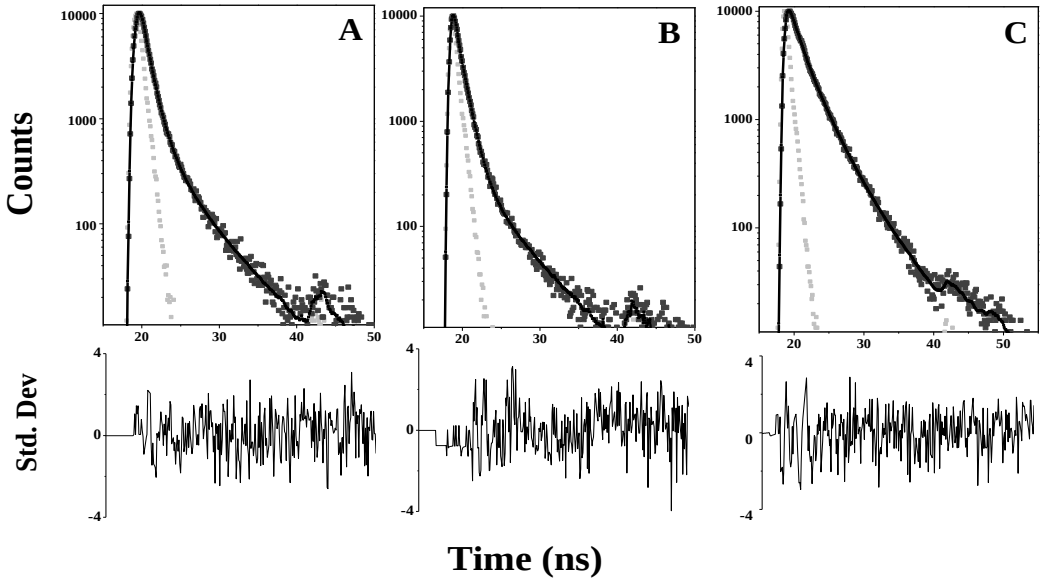


Fig. 4.5. Fluorescence decay profiles of HSP-1/2. (A) Native HSP-1/2, (B) in the presence of 0.15 mM DMPC and (C) upon denaturation with 6 M Gdn.HCl. The fluorescence decay data (■) and lamp profile (■) are shown. Solid lines correspond to nonlinear least square fits of the experimental data to triexponential functions. The lower panels represent residuals.

Table 4.3. Results of life time decay measurements on HSP-1/2. Data given were obtained from triexponential fits. The values for biexponential fits (shown for comparison) clearly indicate that in all cases the fits improved considerably when triexponential fits were chosen.

Sample	α1	α2	α3	τ1	τ2	τ3	τ	<τ>	χ^2 (biexp)	χ^2 (triexp)
HSP-1/2	0.56	0.30	0.13	0.39	1.39	5.11	1.32	3.14	2.03	1.06
+ PrC	0.58	0.37	0.052	0.24	0.75	5.40	0.68	2.46	1.83	1.05
+ DVPC	0.63	0.29	0.077	0.30	1.05	5.20	0.89	2.73	2.24	1.03
+Lyso PC	0.63	0.30	0.071	0.24	1.03	5.96	0.83	3.02	2.52	1.03
+DMPC	0.61	0.3	0.087	0.15	1.04	5.32	0.84	3.01	3.38	1.32
+Gdm.Cl	0.54	0.24	0.25	2.34	0.63	4.84	2.55	3.47	1.51	1.11
+Gdm.Cl-DTT	0.54	0.19	0.28	2.03	0.66	3.99	2.18	2.75	1.36	1.18
+Acryl	0.68	0.26	0.06	0.15	0.8	3.78	0.52	1.91	2.12	1.11
+A-DVPC	0.69	0.27	0.04	0.17	0.68	4.21	0.45	1.76	1.82	1.04

4.4.5. Red Edged Excitation Shift (REES) studies

Plots depicting shift in the fluorescence emission maximum of HSP-1/2 as a function of excitation wavelength under native condition, in the presence of different ligands and upon denaturation with 6 M Gdm.Cl in the absence and in the presence of 10 mM DTT are shown in Fig 4.6. In each case, the excitation wavelength was varied from 280 nm to 307 nm. Fluorescence emission maximum of HSP-1/2 shifted from 338 nm to 341.5 nm upon change in excitation wavelength from 280 to 307 nm, which corresponds to a REES of 3.5 nm. Upon denaturation with Gdm.Cl the emission maximum shifted from 346 nm to 349 nm, which corresponds to a REES of 3 nm. When HSP-1/2 was reduced and denatured the REES decreased to 1 nm with the emission maximum shifting from 347.5 to 348.5 nm upon change in excitation wavelength from 280 to 307 nm. In the presence of PrC and DVPC REES observed was 1 nm, whereas upon to DMPC vesicles a REES of 1.5 nm is observed.

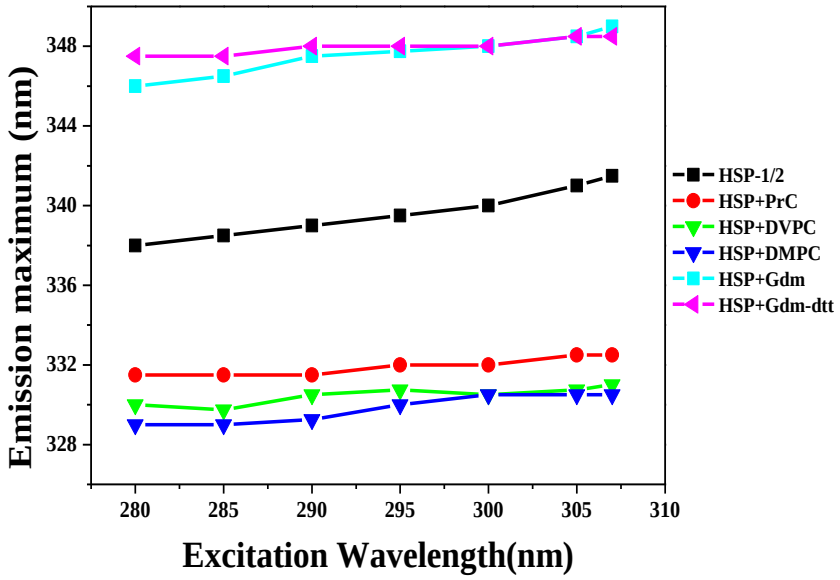


Fig. 4.6. Effect of changing excitation wavelength on the emission maximum of HSP-1/2. (■) Native HSP-1/2, (●) HSP-1/2 + 20 mM PrC, (▼) HSP-1/2 +1 mM DVPC, (∇) HSP-1/2

+0.15 mM DMPC, (□) HSP-1/2 upon denaturation with 6 M Gdm.Cl. and (◀) upon denaturation with 6 M Gdm.Cl and 10 mM DTT.

4.4.6. Chemical denaturation Studies of HSP-1/2

Chemical denaturation of HSP-1/2 by urea, Gdm.Cl and Gdm.SCN was studied by monitoring changes in the intrinsic fluorescence characteristics of the protein. Plots depicting change in emission maximum of HSP-1/2 as a function of denaturant concentration under different conditions are given in Fig. 4.7. From these plots it appears that unfolding of HSP-1/2 with urea, Gdm.Cl and Gdm.SCN under native condition exhibits low cooperativity, since the unfolding curves are non-sigmoidal with no clear midpoint, whereas denaturation of the protein pre-incubated with 10 mM DTT yielded sigmoidal curves with sharp transition for all the three denaturants used indicating cooperative unfolding. Denaturation of reduced HSP-1/2 by Gdm.Cl, Gdm.SCN and urea gave sharp unfolding transitions, with midpoints at 1.5 M, 1.0 M and 3.0 M, respectively, with the corresponding emission maxima being seen at 348 nm, 348 nm and 346.5 nm.

To investigate the effect of ligand binding on the unfolding of HSP-1/2, unfolding experiments were also carried out in presence of 20 mM PrC. Unlike with the native protein, pre-incubation of HSP-1/2 with PrC gave cooperative unfolding curves with Gdm.Cl and Gdm.SCN. The emission λ_{max} shifted from 331 nm to 348 nm for both Gdm.Cl and Gdm.SCN, with transition midpoints centered at 4.3 M and 3.0 M, respectively. Urea did not significantly affect the folding of HSP-1/2 pre-incubated with PrC, with the λ_{max} shifting from 331 to 332.5 nm in presence of 7M urea.

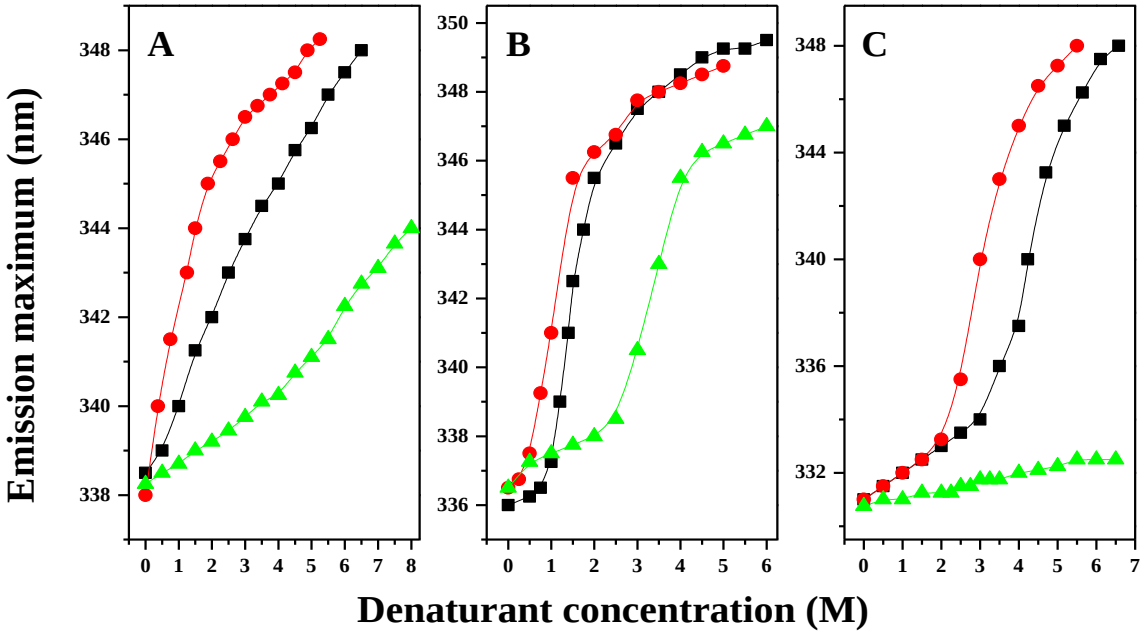


Fig. 4.7. Chemical unfolding of HSP-1/2. Change in emission maximum of HSP-1/2 under different conditions is plotted as a function of denaturant concentration. (A) Native HSP-1/2, (B) HSP-1/2 reduced with 10 mM DTT and (C) HSP-1/2 in presence of 20 mM PrC. The denaturants used are (■) Gdm.Cl, (●) Gdm.SCN and (▲) Urea.

4.5. Discussion

HSP-1/2 is the major protein of equine seminal plasma with implication and importance in the sperm capacitation process and subsequent fertilization. Structurally, HSP-1/2 contains two fibronectin type II domains and an *N*-terminal segment that is intrinsically disordered. It has four tryptophans and 12 tyrosine residues [Calvete et al., 1995]. When excited at 280 nm, fluorescence emission maximum of HSP-1/2 is observed at 338 nm, which indicates that tryptophan residues are the main contributors for the fluorescence emission and are partially buried in the protein matrix. A blue shift in the fluorescence emission maximum along with a significant increase in the emission intensity is observed upon

interaction with choline containing ligands viz., PrC, DVPC and Lyso-PC micelles and DMPC vesicles, indicating that the microenvironment around tryptophan residues becomes more hydrophobic upon ligand binding. The intrinsic fluorescence spectra of HSP-1/2 obtained in presence of PrC and DVPC show a blue shift of 6 nm, which is in good agreement with the results obtained in an earlier study [Greube et al., 2004]. This change in microenvironment around the Trp residues can be due to the removal of water molecules in the vicinity of (or interacting with) the indole side chains of Trp residues. Binding to Lyso-PC micelles and DMPC vesicles results in a blue shift of ~8 nm, these larger shifts can be attributed to the removal of water molecules from the ligand pocket as well as to a partial penetration of segments of the protein containing Trp residues into the hydrophobic core of the lipid vesicles/micelles. It may be noted here that presence of non-choline containing phospholipids such as DMPG and DMPE vesicles led to significantly smaller blue shifts of only 1 and 3 nm, respectively, which is consistent with the specificity of HSP-1/2 for choline phospholipids [Greube et al., 2004].

Intrinsic fluorescence quenching by neutral and charged quenchers gives information regarding the accessibility of Trp residues and nature of the microenvironment around them. Here, quenching experiments carried out with all the three quenchers show that ligand induced conformational changes result in reduced penetration of quencher into the protein matrix. Similar to PDC-109 and PDC-109/B [Anbazhagan et al., 2008; Damai et al., 2009], binding of choline phospholipids to HSP-1/2 resulted in a significant reduction in the accessibility of Trp residues to the quenchers. The linear Stern-Volmer plot for acrylamide quenching of native HSP-1/2 together with a decrease in all the three lifetimes of Trp emission clearly indicates that the quenching process is essentially dynamic in nature. This is unlike PDC-109 and its choline binding domain (PDC-109/B), for

which the Stern-Volmer plots exhibit positive curvature for acrylamide quenching indicating contributions from both static and dynamic quenching components [Anbazhagan et al., 2008; Damai et al., 2009]. Binding of DVPC and DMPC vesicles did not alter the linearity of SV plot, whereas binding of PrC resulted in a biphasic SV plot with acrylamide indicating that binding of PrC induces heterogeneity in the Trp environment. Stern-Volmer plots obtained for both Cs^+ and I^- are biphasic indicating that even though neutral quencher is able to access all the Trp residues equally, the charged quenchers can differentiate the heterogeneity in the microenvironment around Trp residues. These differences can be explained on the basis of partial burial of some of the Trp residues in the protein matrix, due to which they become only partially accessible to the ionic quenchers, whereas the neutral quencher (acrylamide) can access them fully because of its ability to penetrate deeper into the hydrophobic interior of the folded protein. The overall percent quenching for native HSP-1/2 with acrylamide is comparable to that observed for PDC-109. But significant difference is observed in the extent of quenching in the presence of different ligands. With acrylamide, in the presence of PrC and DMPC, HSP-1/2 shows 14.5% and 33.4% less quenching when compared to PDC-109, respectively, indicating that ligand binding results in a stronger shielding of the Trp residues in HSP-1/2 as compared to those in PDC-109. In all the cases percent quenching values obtained in the presence of PrC and DVPC are comparable and this together with the similar REES values for both indicates that the interaction of PrC and DVPC with HSP-1/2 is similar and that the short alkyl chain does not provide any additional shielding as compared to PrC. It is pertinent to note here that at the 1 mM concentration used in the quenching studies, DVPC will be in the monomeric form. Therefore, the additional shielding observed upon binding to DMPC membranes appears to be due to the partial penetration of the protein segments into the membrane interior.

The data presented in Table 4.3 from the lifetime measurements show that unlike PDC-109 which has 5 Trp residues but shows only biexponential decay [Anbazhagan et al., 2008], HSP-1/2 which contains 4 Trp residues shows triexponential decay with two shorter lifetimes and one longer lifetime. This indicates that compared to PDC-109, Trp residues in HSP-1/2 are more heterogeneous in their microenvironment and differ in their spatial interaction with neighboring groups. Upon binding to the lipid moiety, lifetimes of both shorter lifetime components of HSP-1/2 decrease indicating that their environment becomes more hydrophobic, whereas lifetime of the longer component increases. This is unlike PDC-109 where the lifetime of both short and longer lifetime components decreases. These results indicate that upon binding to choline phospholipids, some Trp residues in HSP-1/2 experience more hydrophobic environment probably due to replacement of water molecules with lipid moiety. In addition, Trp residues which are not directly involved in the lipid binding or not present in the ligand binding pocket may be exposed due to conformational changes caused by the ligand binding.

A change in emission maximum (λ_{max}) of 3.5 nm, from 338 to 341.5 nm is observed for the HSP-1/2 under native conditions when excitation wavelength was changed from 280 nm to 307 nm. In general, the emission maximum should not change with change in excitation wavelength because of Kasha's rule, which states that "emission wavelength is independent of excitation wavelength" [Birks, 1970]. But in viscous medium or a condensed phase, where solvent molecules interacting with fluorophore are under motional restriction, polar fluorophores tend to show shift in emission λ_{max} towards longer wavelength when excitation wavelength is increased. Such a phenomenon is termed as Red Edge Excitation Shift (REES) [Mukherjee and Chattopadhyay, 1995]. A REES of 3.5 nm is observed for HSP-1/2 under native condition implies that water molecules (solvent) around Trp residues

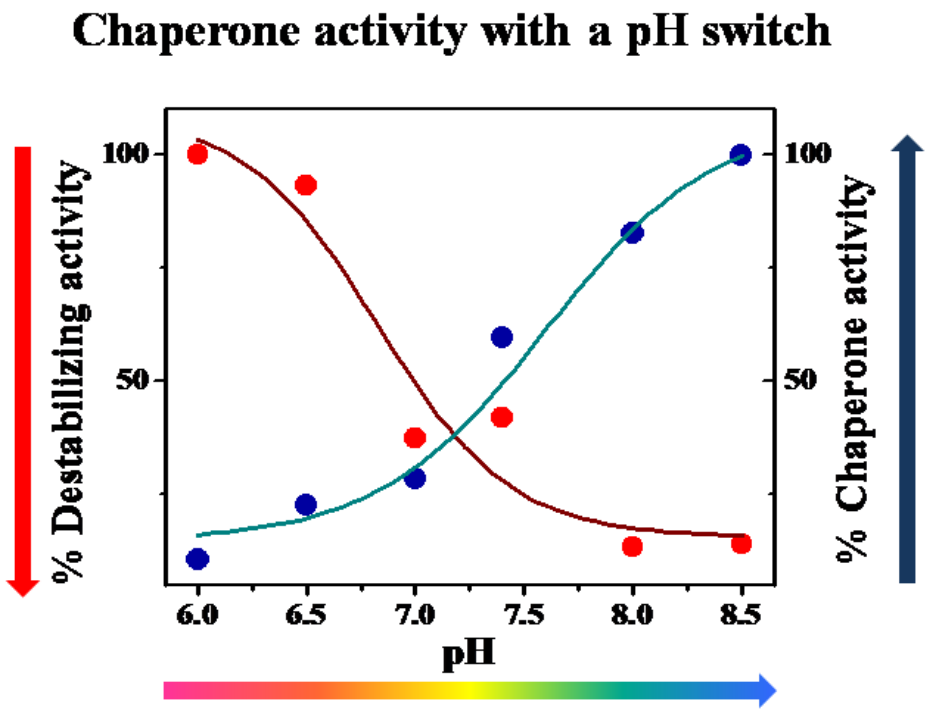
experience motional restriction. As HSP-1/2 has 4 Trp residues this could be an average effect. This is similar to that of PDC-109 which shows a REES of 4 nm. However, the REES observed upon PrC binding is only 1 nm, which is consistent with removal of water molecules surrounding Trp residues. Upon binding to DMPC vesicles a 2 nm decrease in REES is observed compared to native protein. This observation is also similar to that made regarding PDC-109 and can be attributed to a partial penetration of HSP-1/2 into the DMPC membrane hydrophobic core as HSP-1/2 is known to penetrate in to the lipid membrane interior. It has been shown that the rate of solvent reorientation/relaxation in membranes is depth dependent, wherein the REES observed decreases with increasing depth of penetration of the fluorophore into the hydrophobic core of the membrane [Chattopadhyay and Mukherjee, 1999a, b]. Reduction in REES upon binding to DMPC membrane is 2.5 and 2 nm in PDC-109 and HSP-1/2, respectively, suggesting that Trp residues in HSP-1/2 are less embedded in lipid interior as compared to PDC-109. These results are consistent with the results of previous EPR studies [Greube et al., 2004]. The overall REES observations are very similar to that of PDC-109 indicating similar hydration/ tertiary structure of HSP-1/2 and PDC-109.

Intrinsic fluorescence of Trp residues in proteins is very sensitive to the microenvironment surrounding the indole side chains. Any major changes seen in the fluorescence intensity or emission maximum can be due to two factors: i) quenching (or reversal of quenching) by solvent molecules or neighboring groups in the three dimensional structure of protein, ii) the solvent relaxation surrounding the Trp residue which determines the magnitude of Stokes shifts. The first factor is responsible for signal intensity and the latter to emission maximum [Vivian and Callis, 2001; Duy and Fitter, 2006]. Chaotrope-induced unfolding of a protein results in changes in both the factors mentioned above. When unfolding of HSP-1/2 by denaturants such as Gdm.Cl, Gdm.SCN and urea was investigated, the

fluorescence intensity was found to change, with a concomitant red shift in emission maximum. As shown in Fig. 4.7A, denaturation of HSP-1/2 did not yield sigmoidal unfolding curves, indicating poor cooperativity of the transition, which is atypical of globular proteins. While the emission λ_{max} shifted from 338 to 347 nm in presence of 6M Gdm.Cl, the REES value decreased only marginally to 3 nm, suggesting that the water molecules are still motionally restricted under denaturing condition. These observations indicate that Trp residues are nearly completely exposed to the polar environment but are still in some kind of stable residual structure resulting only partial unfolding of HSP-1/2. This peculiar behavior of non-cooperative partial unfolding can be because of presence of three domains (two Fn-II domains and one flanking region) [Calvete et al., 1995] in the structure which may unfold separately or may be due to the polydispersity of the protein [Calvete et al., 1997] or a combination of both these factors. The reduction in disulphide bonds has caused the protein to denature with unfolding midpoints centered at 1.5 M of Gdm.Cl. REES of only 1 nm is observed for the reduced HSP-1/2 under denatured conditions. These results indicate that reduction of disulfide bonds allows the protein to undergo cooperative unfolding. Surprisingly, a broad unfolding transition curve was observed when unfolding experiments were carried out with Gdm.Cl and Gdm.SCN on HSP-1/2 pre-incubated with PrC. It is known that in the presence of PrC, the polydisperse nature of the HSP-1/2 is lost and predominantly monomeric species exists in solution [Calvete et al., 1997]. This was also attributed to the loss of chaperone like activity of HSP-1/2 [Sankhala et al., 2012]. Cooperative unfolding observed in the presence of PrC indicates that, polydispersity may be responsible the protein exhibiting non-cooperative unfolding under native conditions, because different oligomeric species may be unfolding simultaneously with different degrees of unfolding at a given concentration of the denaturant.

In summary, intrinsic fluorescence studies provide additional evidence for the specificity of HSP-1/2 towards choline phospholipids. Quenching studies indicate that binding of phosphorylcholine and choline phospholipids results in a shielding of Trp residues, which is similar to the observations made in case of PDC-109. Comparison of the quenching parameters and REES values of HSP-1/2 in presence of PrC, DVPC and DMPC indicate that while the short alkyl chain of DVPC may not contribute much towards binding, the long hydrocarbon chain of DMPC makes a significant positive contribution. Compared to PDC-109, slight decrease in the changes in the REES is observed on binding to DMPC vesicles to HSP-1/2. This together with earlier studies indicates that HSP-1/2 is less effective in penetrating into the membrane core. This can be a reason for the inability of HSP-1/2 to induce cholesterol efflux, *in vitro*. Unfolding studies on HSP-1/2 indicate that presence of disulphide bonds, ligand binding and polydispersity modulate the cooperativity of the unfolding transition of the protein.

A pH switch regulates the inverse relationship between membranolytic and chaperone-like activities of HSP-1/2



The work presented in this chapter is communicated

Chapter 5

5.1. Summary

HSP-1/2, a major protein of horse seminal plasma displays membranolytic and chaperone-like activities. Here we show that lytic activity of HSP-1/2 is high at mildly acidic pH (6.0-6.5) and low at mildly basic pH (8.0-8.5) whereas the chaperone like activity is high at mildly basic pH and low at mildly acidic pH, that is, these two activities have an inverse relationship regulated via a pH switch, which is reversible. The higher chaperone-like activity observed at mildly basic pH could be correlated to an increase in surface hydrophobicity of the protein.

Chapter 5

5.2. Introduction

The stability and function of a protein depend mainly on its correct folding and the environment around it. Any significant changes in the environment of a protein can lead to its mis/unfolding and result in diseases such as Alzheimers, Parkinsons etc. In order to adapt to these kinds of "stress" conditions cells have evolved chaperone machineries, which consist of specific proteins that assist in correct folding of the nascent and partially mis/unfolded proteins as well as specific proteases, which degrade the mis/unfolded proteins [Lindquist and Craig, 1998]. A diverse class of ATP dependent chaperones called *heat shock proteins* (Hsps) operate inside the cell, regulating protein folding/unfolding [Hendrick and Hartl, 1993]. Recently several extracellular chaperones have been identified; these are termed *small heat shock proteins* (sHsps), which are ATP independent and act non-specifically on a variety of target proteins. Surface hydrophobicity and polydispersity have been shown to be important for the chaperone-like activity (CLA) of these proteins [Bakthisaran et al., 2015; Bova et al., 1997; Delbecq et al., 2015].

In recent work, we have shown that two homologous fibronectin type-II (FnII) proteins from mammalian seminal plasma, PDC-109 (bovine) and HSP-1/2 (horse), exhibit CLA *in vitro* [Sankhala and Swamy, 2010; Sankhala et al., 2012]. The horse seminal plasma protein, HSP-1/2 is a non-separable mixture of two proteins with the same primary sequence but with different degrees of glycosylation and lengths of the *N*-terminal segment [Calvete et al., 1995]. These FnII proteins recognize a variety of ligands [Plante et al., 2015] among which their recognition of choline-phospholipids is biologically significant since it mediates their interaction with spermatozoa [Desnoyers and Manjunath, 1992; Swamy, 2004]. Binding of these proteins to the sperm cells induces an efflux of cholesterol

and choline phospholipids from the plasma membrane (termed *cholesterol efflux*), a crucial step in the sperm capacitation process, which primes the sperm cell to fertilize the egg [Manjunath and Theréin, 2002]. Incubation of sperm cells and liposomes containing phosphatidylcholine with PDC-109 resulted in membrane perturbation and formation of lipoprotein complexes and nano-tube like structures [Moreau and Manjunath,1999; Gasset et al., 2000; Damai et al., 2010; Ramakrishnan et al., 2001; Lafleur et al., 2010]. Crystal structure of PDC-109 complexed with phosphorylcholine (PrC), the head group moiety of choline phospholipids, has shown that each FnII domain of PDC-109 binds to one ligand molecule and both binding pockets are on the same face of the protein [Wah et al., 2002]. Binding of PDC-109 to model membranes destabilizes the membrane structure by extracting lipids from the outer leaflet; presence of cholesterol in the membrane reduces this disruption [Tannert et al., 2007; Swamy et al., 2002]. FnII proteins are also found to be involved in the formation of oviductal sperm reservoir and regulate sperm cell volume [Sahin et al., 2009; Ignotz et al., 2007]. Studies on the interaction of HSP-1/2 with model membranes using EPR and fluorescence spectroscopy have shown that HSP-1/2 induces membrane perturbation by intercalating into the hydrophobic core of the membrane, albeit to a lesser extent as compared to PDC-109 [Greube et al., 2004]. While HSP-1/2 has been reported to be less effective in lipid extraction compared to that of PDC-109, to date there have been no studies investigating the effect of HSP-1/2 on the structural integrity of the model membranes. In the present study, the effect of HSP-1/2 binding on cell and supported membranes along with several factors affecting it has been investigated using various approaches.

5.3. Materials and Methods

5.3.1. Materials

Bis-ANS, choline chloride calcium salt, phosphorylcholine chloride calcium salt (PrC) and heparin-Agarose type-I beads were purchased from Sigma (St. Louis, MO, USA). Dimyristoylphosphatidylcholine (DMPC) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). *p*-Aminophenyl phosphorylcholine-Agarose (PPC-Agarose) column was obtained from Pierce Chemical Co. (Oakville, Ontario, Canada). All other chemicals were obtained from local suppliers and were of the highest purity available.

5.3.2. Purification of HSP-1/2

HSP-1/2 was purified from seminal plasma collected from healthy horses as described earlier [Sankhala et al., 2012]. Briefly, seminal plasma was subjected to affinity chromatography on heparin-Agarose and *p*-aminophenyl phosphorylcholine columns followed by reverse phase HPLC (RP-HPLC). The HSP-1/2, thus obtained, was extensively dialyzed against 50 mM tris buffer, pH 7.4, containing 0.15 M NaCl and 5 mM EDTA (TBS) and its purity was checked by SDS-PAGE. HSP-1/2 was further concentrated using Millipore Amicon filters (10 kDa cutoff) and a stock of 1 mg/mL was used for further experiments. For pH dependent studies HSP-1/2 was dialyzed against 50 mM phosphate buffer containing 150 mM NaCl for pH 6.0-7.0. For studies in the pH range 7.4-8.5, 50 mM Tris buffer containing 150 mM NaCl was used.

5.3.3. Atomic force microscopic studies

Supported membranes of DMPC were prepared as reported earlier [Damai et al., 2010]. DMPC (0.2 mg) was taken in a glass test tube and dissolved in chloroform or chloroform containing a trace of methanol. The solvent was then dried under a gentle stream of nitrogen gas followed by vacuum desiccation for 3-4 h. The dry lipids were hydrated with TBS to give desired concentration and

subjected to sonication in a bath sonicator for 30 minutes. Sonicated vesicles were deposited gently on a freshly cleaved mica sheets or a clean glass cover slips (Thermo-Fisher) and left at room temperature for 30 minutes. Samples were then washed with HPLC grade water, dried and transferred to AFM stage for imaging. To investigate the effect of HSP-1/2 on membrane formation, 100 μg of HSP-1/2 was added to the sonicated lipid vesicles before their deposition on mica or glass and experiments were carried out in air. Imaging was performed in semi-contact mode by using NSG10 cantilevers with silver reflective coating and a spring constant of 11.8 N/m. Force was kept as low as possible by constantly adjusting the set-point and feedback gain during imaging. Images obtained were analyzed using NOVA software, supplied by NTMDT.

5.3.4. Absorption spectroscopy

Erythrocyte membrane disruption assay was performed as described earlier [Damai et al., 2010]. In experiments aimed at investigating the concentration dependence of HSP-1/2 on membrane disruption, 100 μL of 4% human RBC suspension in TBS was incubated with varying amounts of the protein and the final volume was adjusted to 0.5 mL with TBS. The reaction mixture was then incubated for 1 hour before centrifuging the samples at 1500 × g for 5 minutes. Supernatant was collected and checked for absorbance at 415 nm corresponding to haem moiety using an Agilent Cary-100 UV-Vis spectrophotometer. For investigating the kinetics of erythrocyte membrane disruption, 50 μg of HSP-1/2 was incubated with 4% RBC suspension in different vials and incubated for different time intervals before measuring absorbance as described above. To investigate the effect of ligands on erythrocyte lysis, HSP-1/2 was incubated with PrC or ChCl at different concentrations prior to its addition to the erythrocyte suspension and the experiment was carried out as described above. For pH

dependent studies, 100 μL of 4% erythrocyte suspension in TBS was taken and 400 μL of buffer of desired pH was added and centrifuged at 1500 × g for 5 minutes. The clear supernatant was decanted and total volume was made up to 500 μL with buffer of desired pH. This was repeated two times such that erythrocytes were in the buffer of appropriate pH and then the assay was carried out as described above. Results from a minimum of three independent experiments have been presented together with standard deviations.

Aggregation assays to investigate the chaperone like activity of HSP-1/2 were performed as described previously [Sankhala et al., 2012]. Briefly, target proteins ADH (0.05 mg) or aldolase (0.1 mg) in buffers of different pH, were mixed with HSP-1/2 in the same buffer for 5 minutes before incubating at 48 °C. Heat induced aggregation of the target proteins was monitored by increase in the sample turbidity at 360 nm as a function of time. Aggregation of native ADH or aldolase was taken as 100% and aggregation of other samples was normalized with respect to it. Results from three independent experiments have been presented along with standard deviations.

5.3.5. Confocal microscopy

HSP-1/2 induced destabilization of human erythrocyte membrane integrity and morphology was investigated by confocal microscopy. Imaging was done in transmission mode using a Leica TCS SP2 confocal microscope (Heidelberg, Germany). A 0.04% suspension of human erythrocyte in TBS was used as the control. To investigate the effect of HSP-1/2, the erythrocyte suspension was incubated with HSP-1/2 (100 μg/mL). Then 50 μL aliquots of the mixture were taken at 45 and 90 minutes, spotted on a clean glass slip (Thermo Fisher) and transferred to confocal stage for imaging. To investigate the effect of ligand

binding, HSP-1/2 was pre-incubated with 20 mM PrC before incubating with the erythrocyte suspension and imaging was performed after 90 minutes.

5.3.6. Circular dichroism spectroscopy

Circular dichroism spectroscopic studies were performed using an AVIV 420SF CD spectrometer (Lakewood, NJ, USA), fitted with a thermostatted cell holder and a thermostatic water-bath with a scan speed of 20 nm/min. Far-UV spectra were recorded at a protein concentration of ~0.1mg/mL. Spectra were recorded using a 2 mm path length quartz cell and each spectrum reported was the average of 3 consecutive scans from which buffer scans, recorded under the same conditions, were subtracted.

5.3.7. Steady-state fluorescence measurements

Steady-state fluorescence experiments were carried out using a Spex Fluoromax-3 fluorescence spectrometer at room temperature with excitation and emission band pass filters set at 3 and 5 nm, respectively. bis-ANS was first dissolved in methanol and a stock solution of 250 μM was prepared by diluting the methanolic solution with buffer of appropriate pH. From this an aliquot of bis-ANS was added to a solution containing HSP-1/2 and the volume was adjusted to 1.0 mL with the buffer. The content of methanol was <1% for all samples. The final concentration of bis-ANS was 5 μM and that of the protein was 2.8 μM. After a 3 minute incubation period, samples were excited at 395 nm and emission spectra were recorded between 410 and 600 nm. Spectra of bis-ANS alone, obtained in buffers of different pH were subtracted from the corresponding spectra recorded in the presence of HSP-1/2 before further analysis. The results given are the averages of at least two independent experiments (with standard deviations of <5% in all cases).

5.4. Results and Discussion

5.4.1. Atomic Force Microscopic (AFM) studies

AFM images depicting the effect of HSP-1/2 on the morphology/integrity of supported model DMPC membranes are shown in Fig 5.1. Upon deposition on either mica or on a glass cover slip, sonicated vesicles of DMPC formed well ordered supported membrane multilayers, whereas in presence of HSP-1/2 no membranous structure was observed. Instead a uniform distribution of small spherical aggregate-like structures with diameter of ~60-140 nm were observed with an average mean size of ~80 nm. This is very similar to the observations made for the interaction of PDC-109 with model membranes and sperm plasma membrane where lipoprotein aggregates with mean size of ~80-90 nm were observed [Moreau and Manjunath,1999; Gasset et al., 2000; Damai et al., 2010; Ramakrishnan et al., 2001], and suggest that the above aggregates obtained with HSP-1/2 are also lipoprotein particles.

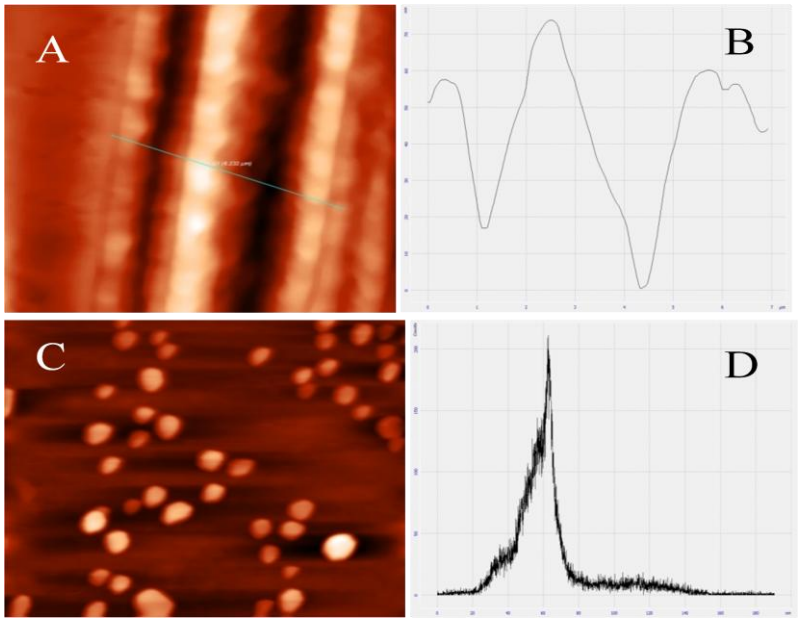


Fig. 5.1. Effect of HSP-1/2 on supported model membrane studied by AFM. A) AFM image of DMPC multilayers in air (7 × 7 μm). B) Height profile of region corresponding to blue bar marked in A. C) DMPC in the presence of 0.25 mg/ml of HSP-1/2 (5 × 5 μm). D) Distribution density histogram analysis for C.

These results indicate that HSP-1/2 acts in a manner similar to PDC-109 with regard to destabilization of cell and model membranes.

5.4.2. Erythrocyte lysis assay

Human erythrocytes were taken as model cells to investigate the effect of HSP-1/2 binding on the cell membrane as the lipid composition of erythrocytes and equine spermatozoa are quite similar, with both having high content of cholesterol and choline phospholipids [Virtanen et al., 1998; Parks and Lynch, 1992]. The results obtained from these experiments show that HSP-1/2 binding destabilizes the RBC in a time- and concentration-dependent manner. The destabilization ability (measured by monitoring A_{415}, which corresponds to release of haem due to disruption of the erythrocyte plasma membrane) of HSP-1/2 increased in a sigmoidal fashion and reached saturation at 75 μg/mL and then levelled off (Fig. 5.2D). Kinetics of destabilization in the presence of 100 μg/mL of HSP-1/2 shows time dependent increase in absorption up to 150 min and then remains constant (Fig 5.2E). Previously Greube et al' [2004], reported that HSP-1/2 was ineffective in removal of phospholipids and cholesterol from the RBC membrane, but they did not indicate whether binding of the protein resulted in any lysis of the cells. Therefore, it is not possible to correlate our results with those of Greube et al.

The time dependent disruption of RBC was further confirmed by confocal microscopy. Human erythrocytes under normal conditions are 6-9 μm in diameter with their cell membrane intact (Fig. 5.2A). When incubated with 100 μg/mL of HSP-1/2 for 45 min, partially disrupted erythrocytes along with decrease in total

number of cells was observed (Fig. 5.2B). After incubation for 90 minutes, only fragmented cell membranes and very few cells with intact membrane were observed (Fig. 5.2C), indicating time dependent membrane disruption by HSP-1/2.

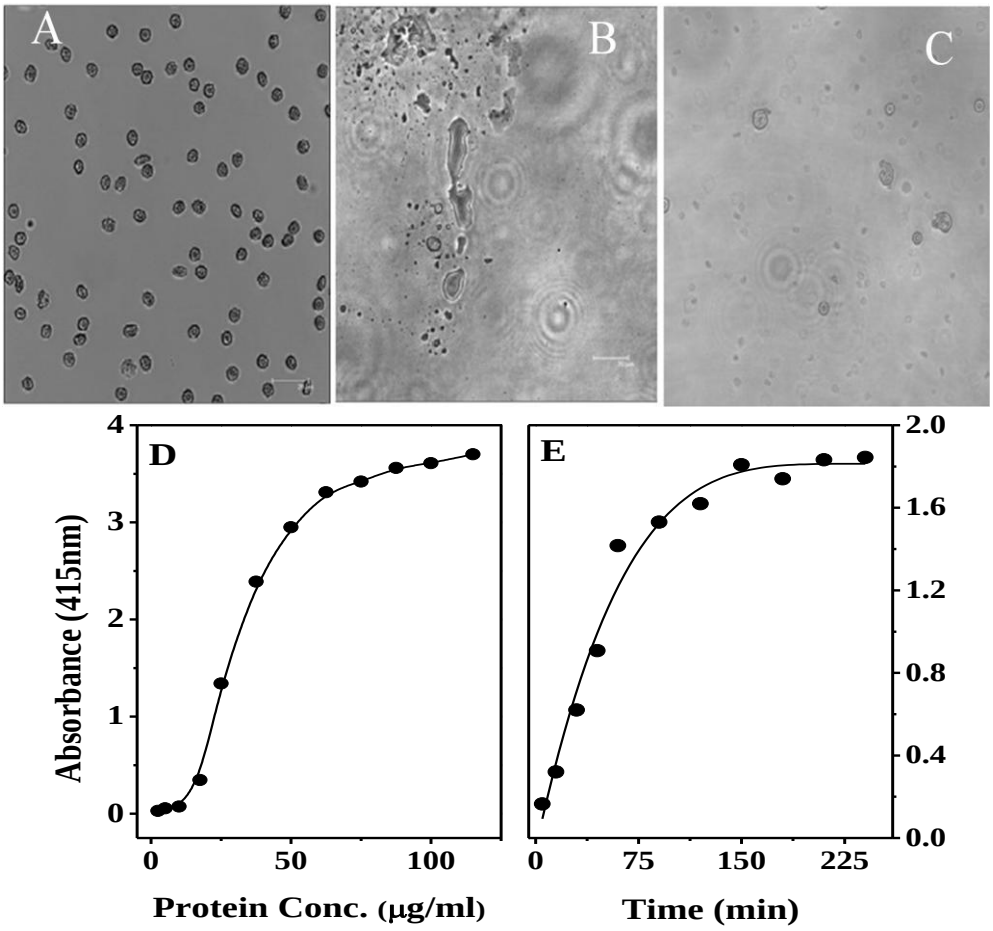


Fig. 5.2. Erythrocyte membrane destabilization by HSP-1/2. Confocal images of human erythrocytes under different conditions are shown: (A) in buffer alone, (B) upon incubation for 45 min with 100 μg/mL HSP-1/2, (C) upon incubation for 90 min with 100 μg/mL HSP-1/2. D) Effect of increasing the concentration of HSP-1/2 on erythrocyte lysis. E) Kinetics of erythrocyte lysis in the presence of 100 μg/mL HSP-1/2.

5.4.3. Effect of ligands and pH on erythrocyte lysis

To investigate the effect of ligand binding on erythrocyte lysis by HSP-1/2, the lysis assay was performed in the presence of PrC. When HSP-1/2 was preincubated with PrC its ability to destabilize the erythrocyte plasma membrane decreased with increase in PrC concentration, clearly indicating that the interaction of HSP-1/2, medicated by choline phospholipids present on the erythrocyte membrane is responsible for the membrane disruption (Fig. 5.4A). Choline chloride (ChCl), which is devoid of the phosphate group, was less effective in preventing the erythrocyte lysis. Confocal microscopy confirmed that addition of HSP-1/2 pre-incubated with 20 mM PrC to the erythrocytes did not significantly affect their morphology (Fig. 5.3) These results demonstrate that erythrocyte membrane lysis is a specific effect caused due to choline phospholipid-HSP-1/2 interaction.

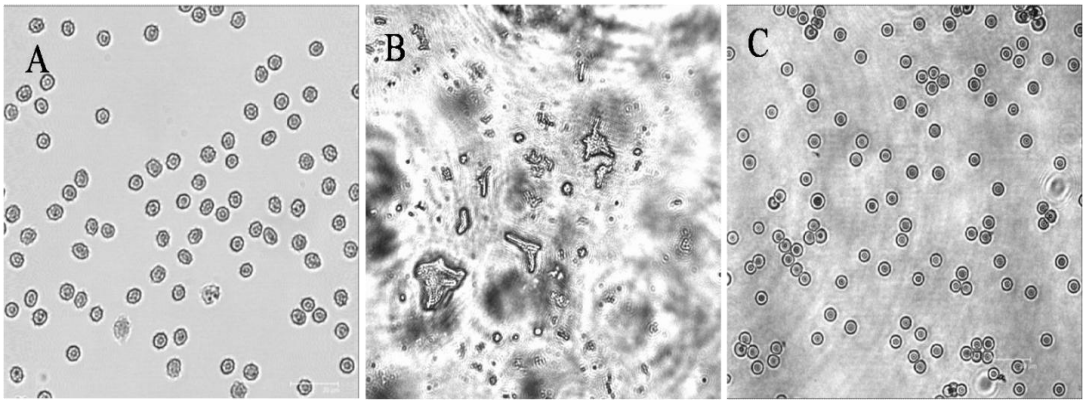


Fig. 5.3. Disruption of erythrocyte membrane by HSP-1/2 and protection by PrC studied by confocal microscopy. Images shown correspond to human erythrocytes (A) in TBS buffer, (B) after 90 min incubation with 100 μg/mL HSP-1/2, and (C) after 90 min incubation with 100 μg/mL HSP-1/2 that was pre-incubated with 20 mM PrC.

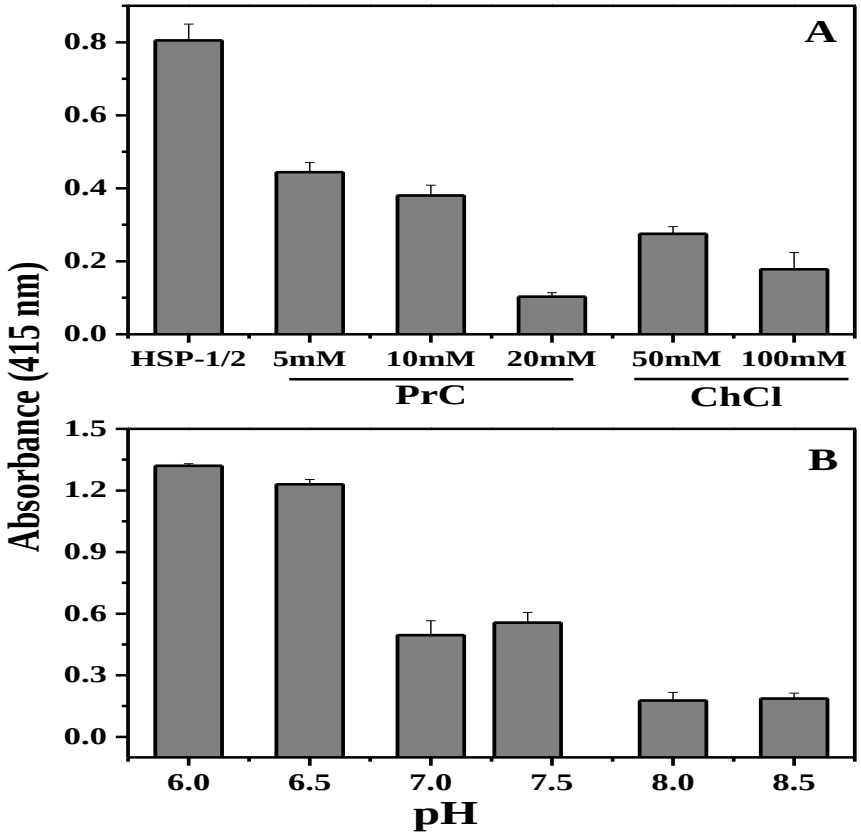


Fig. 5.4. Effect of different conditions on erythrocyte membrane destabilization by HSP-1/2. A) Effect of concentration of ligand preincubated with HSP-1/2; phosphorylcholine and choline chloride. B) Effect of pH of the medium.

pH variation is an important factor in the reproductive tract as it is correlated to hyperactivity of sperm, protein tyrosine phosphorylation and intracellular calcium movement [Kirichok and Lishko, 2011; McPartlin et al., 2007]. To investigate the effect of pH on the membrane destabilizing ability of HSP-1/2, we performed the erythrocyte lysis assay at different pH between 6.0 and 8.5. Interestingly, the ability of HSP-1/2 to destabilize erythrocyte cell membrane was found to decrease as the pH is increased (Fig. 5.4B). HSP-1/2 induced lysis was ~110% higher at pH 6.0 and 6.5 as compared to that observed at the

physiological pH of 7.4. On the other hand, significant decrease in erythrocyte lysis was observed at mildly basic conditions with only 30 and 32% lysis being observed at pH 8.0 and 8.5, respectively, as compared to that seen at pH 7.4.

5.4.4. Effect of pH on chaperone-like activity of HSP-1/2

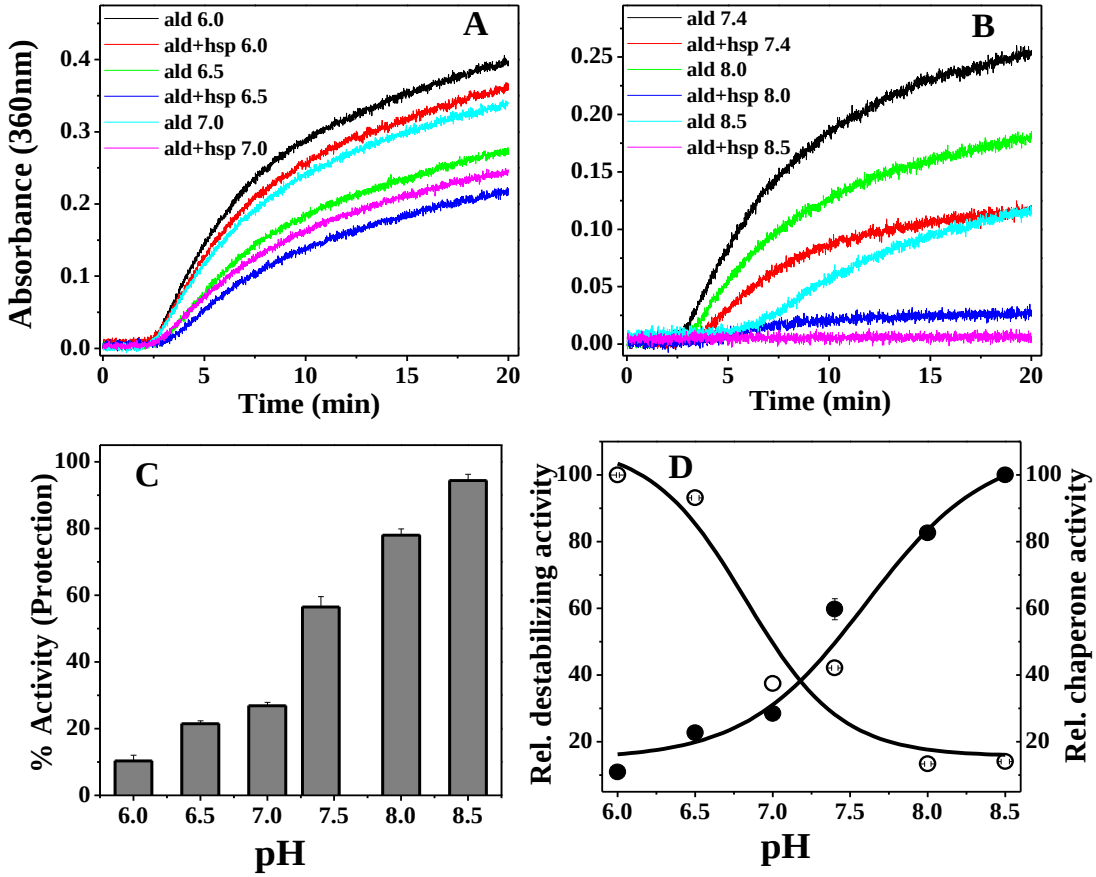


Fig. 5.5. Effect of pH on the CLA of HSP-1/2. Aggregation profiles of aldolase (0.1mg/mL) alone and in the presence HSP-1/2 (0.15mg/mL) at different pH. A) At pH 6.0, 6.5 and 7.0. B) At pH 7.4, 8.0 and 8.5. C) Bar diagram showing % protection offered by HSP-1/2 at different pH. D) pH dependence of erythrocyte membrane destabilizing and chaperone-like activities.

In further studies, aimed at investigating the effect of pH on the chaperone-like activity of HSP-1/2, we performed aggregation assays with ADH and aldolase as target proteins in order to probe the protection offered by HSP-1/2 against thermal stress [Sankhala et al., 2012]. As shown in Fig. 5.5A & B, aldolase alone shows significant aggregation when incubated at higher temperatures and in presence of HSP-1/2 this aggregation is decreased. CLA was measured as percent protection offered by HSP-1/2 as compared to target protein alone. Interestingly, HSP-1/2 at pH 8.5 showed highest CLA and this has been taken as 100% and other values were normalized with respect to this. In comparison, 83% and 60% activity was observed at pH 8.0 and 7.4, respectively, whereas at the neutral pH of 7.0, only ~28% activity was observed. At mildly acidic pH of 6.5 and pH 6.0 the CLA decreased to ~23% and 11%, respectively. Similar results were obtained when ADH was used as the target protein (Fig. 5.6) These results indicate that the CLA of HSP-1/2 increases significantly at mildly basic conditions, whereas it is drastically reduced at mildly acidic conditions. These results imply that the CLA of HSP-1/2 is inversely related to its membrane destabilizing activity and this dual functionality of HSP-1/2 is regulated by a pH switch.

In case of a few chaperone proteins which also exhibit another biological activity, it was reported that the latter activity decreases with a concomitant increase in the CLA or vice versa, when the protein is subjected to certain extreme conditions such as high temperature or oxidative stress. The dual functionality of such chaperone proteins is said to be regulated by a temperature or redox switch [Spiess et al., 1999; Jakob et al., 1999; Hong et al., 2005]. For example, DegP which functions as a chaperone at low temperature exhibits strong proteolytic activity at high temperature [Spiess et al., 1999], whereas the CLA of Hsp33 is redox regulated with the chaperone activity of the protein becoming activated

under oxidizing conditions [Jakob et al., 1999]. In case of HSP-1/2 the inverse relation between membranolytic activity and CLA are regulated by moderate changes of pH near the physiological range (pH 6.0-8.5). This novel mode of regulating two different functional activities of a protein appears to be of considerable significance in view of the widespread occurrence of FnII proteins, especially in the seminal plasma of mammals [Plante et al., 2015; Ozhogina et al., 2001].

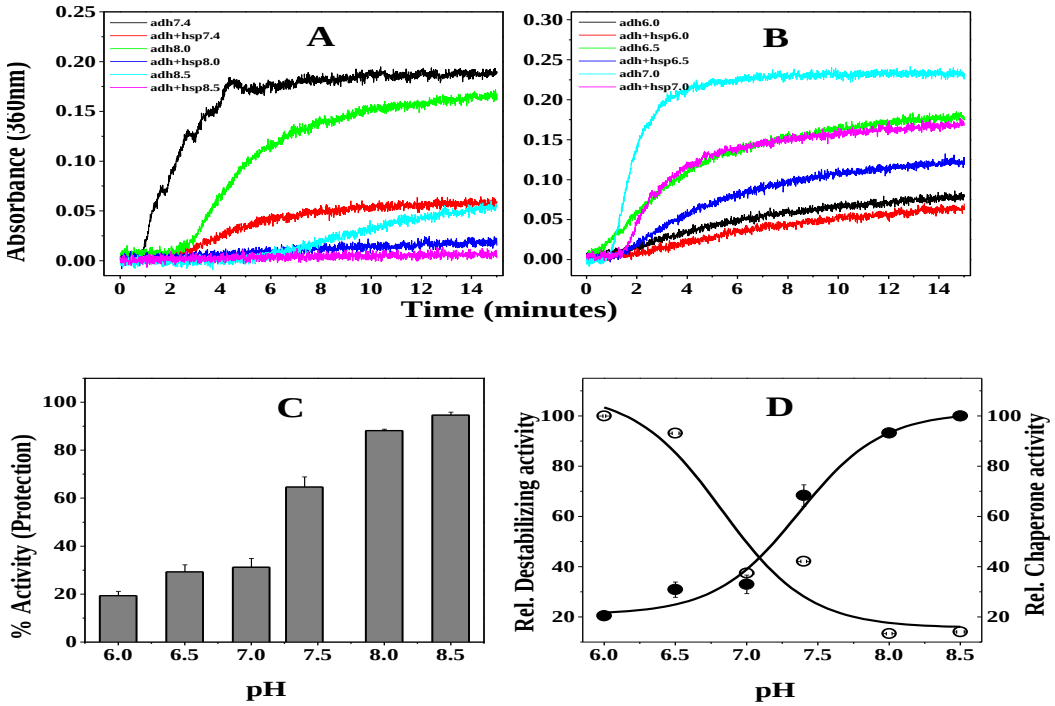


Fig. 5.6. Effect of pH on the chaperone like activity of HSP-1/2. Aggregation profiles of ADH alone and in the presence HSP-1/2 at different pH are shown. A) At pH 6.0, 6.5 and 7.0. B) At pH 7.4, 8.0 and 8.5. C) Bar diagram showing % protection offered by HSP-1/2 at different pH. D) pH switch depicting relative erythrocyte membrane destabilizing and relative CLA.

Since the pH of mammalian seminal plasma is mildly basic in nature, the reduced cell membrane destabilization by HSP-1/2 at mildly basic conditions is physiologically relevant as it prevents premature destabilization of sperm plasma membrane and hence premature capacitation before reaching the egg. The high CLA of HSP-1/2 is also significant as the sperm cells and related proteins encounter hostile environment in the female reproductive tract in the early stages. In the female reproductive tract, the intracellular pH of the oviduct is reported to be ~7-8, which is correlated with protein tyrosine phosphorylation, an important step in the maturation of stallion spermatozoa. Time dependent rise in the pH was observed in the oviduct-bound sperm from 6.8 to 7.8 [Goudet, 2011]. Binding of sperm to oviduct was shown to reduce sperm motility, inhibit capacitation and increase sperm survival [Leemans et al., 2014; González-Fernández et al., 2012]. Thus the inhibition of sperm capacitation in the alkaline conditions can be due to low membrane destabilizing activity of sperm-bound HSP-1/2. In seminal plasma, HSP-1/2 is present in membrane bound form as well as in the form of polydisperse oligomers, which interact with other proteins of the seminal plasma [von Fellenberg et al., 1985]. The low membrane destabilizing ability at basic pH of the membrane-bound HSP-1/2 is relevant to maintaining the oviductal reservoir of uncapacitated spermatozoa, whereas the high CLA of the unbound fraction helps the other proteins of the seminal plasma to counter the hostile conditions that are encountered in female reproductive tract.

5.4.5. Reversibility of pH switch

To investigate whether the pH dependent activity switch from higher membrane destabilization at acidic pH to high CLA at basic pH is reversible, HSP-1/2 at pH 6.5 was dialyzed against pH 8.5 buffer and checked for CLA and erythrocyte lysis. The samples were again dialyzed back to pH 6.5 and both activities were assessed.

As shown in Fig. 5.7 the results obtained show that while the membranolytic activity exhibits a decrease at pH 8.5, the CLA shows a strong increase at the basic pH. Importantly, upon changing the pH of the medium back to 6.5, both CLA and erythrocyte destabilizing activity of the protein become comparable to those seen originally at pH 6.5. These observations suggest that the pH switch regulating the dual functionality of HSP-1/2 is reversible.

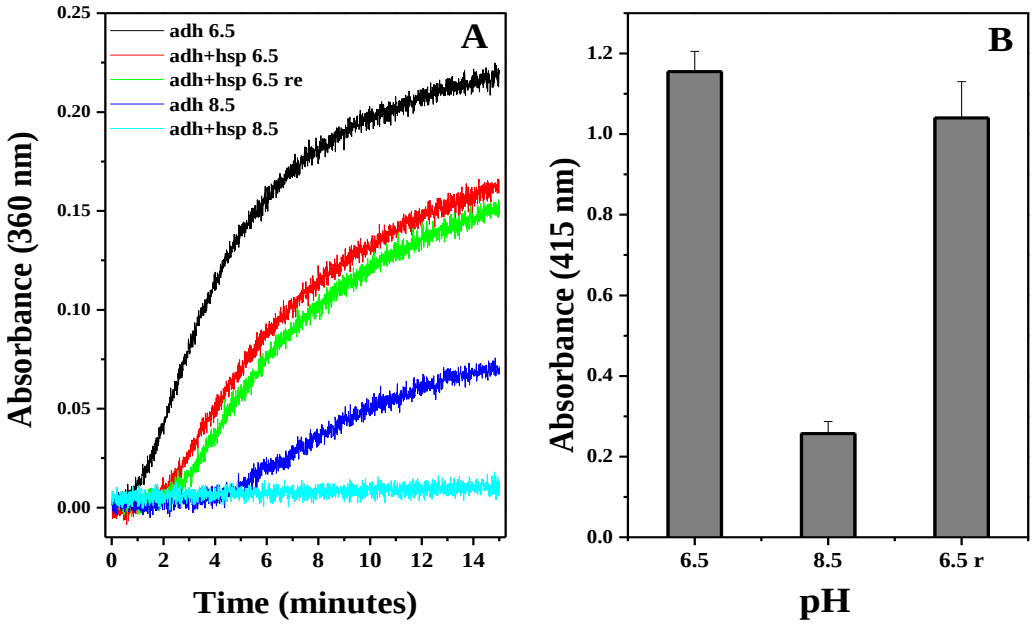


Fig. 5.7. Reversibility of CLA and membranolytic activities of HSP-1/2 with pH change. A) Aggregation profiles of ADH alone and in the presence of HSP-1/2 at pH 6.5, 8.5 and upon re-dialysis to pH 6.5 (6.5r). B) Erythrocyte lysis assay in the presence of HSP-1/2 at pH 6.5, 8.5 and upon redialysis to pH 6.5.

5.4.6. Structural changes of HSP-1/2 associated with pH change

Changes in the structural features and surface hydrophobicity of HSP-1/2 due to changing the pH of the medium were investigated using circular dichroism (CD) and fluorescence spectroscopy. Far-UV CD spectra of HSP-1/2 recorded at different pH are qualitatively similar with some variation in the spectral intensity

(Fig. 5.8). Although the data could not be analyzed because of lack of a suitable reference data set [Gasset et al., 1997], the differences in the spectral intensity suggest that some changes take place in the structural features of the protein with change in pH. HSP-1/2 shows a broad positive band at ~224 nm and a shoulder at~212 nm, which is characteristic of native FnII proteins [Sankhala et al., 2012; Gasset et al., 1997]. These bands suggest that non-peptide factors such as disulphide bonds and aromatic residues and coupling between them are the main contributors for the CD signal [Gasset et al., 1997; Strickland, 1974]. The spectral intensity is found to decrease with increase in pH suggesting small decrease in the total structural content of the protein and/or changes in the orientation of chiral components and coupling between them.

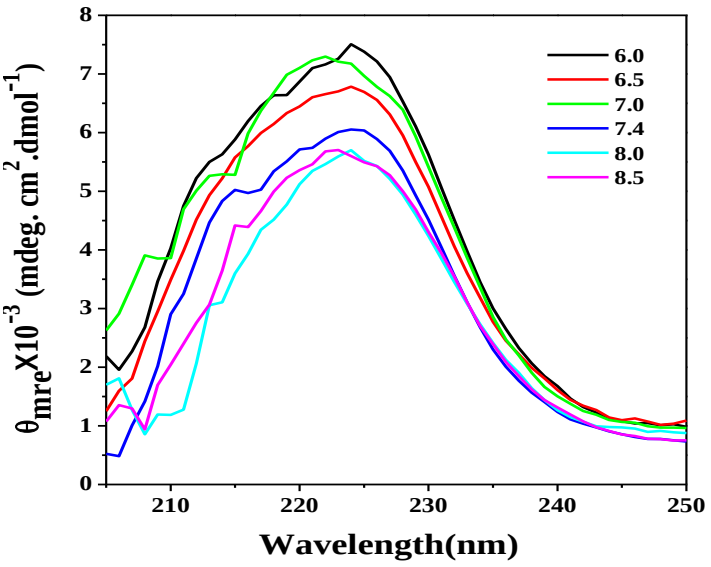


Fig. 5.8. Far-UV circular dichroism spectra of HSP-1/2 at different pH.

In further studies, we investigated pH induced changes in the hydrophobicity of HSP-1/2 using fluorescence spectroscopy, by monitoring the fluorescence emission characteristics of the hydrophobic probe, bis-ANS. While

only a marginal increase in the fluorescence intensity of bis-ANS bound to HSP-1/2 was observed when pH was increased from 6.0 to pH 7.4, further increase in the pH to 8.0 and 8.5, resulted in 1.3 fold and 1.7 fold increase in fluorescence intensity, respectively, indicating that the surface hydrophobicity of the protein increases significantly at basic pH (Fig. 5.9). Surface hydrophobicity is an important factor and is essential for many chaperones to exhibit CLA [Hong et al., 2005; Moparthi et al., 2010; Sheluho and Ackerman, 2001], as the most accepted mechanism of chaperone action is that the chaperone interacts with partially unfolded target proteins through hydrophobic interactions [Hlodan and Hartl, 1993; Lee et al., 2007. Larger hydrophobic surface results in higher interaction with target protein which in turn results in high CLA. The high hydrophobicity exhibited by HSP-1/2 at mildly basic conditions correlates well with the high CLA and indicates that hydrophobicity is an important factor for the chaperone-like activity of this protein.

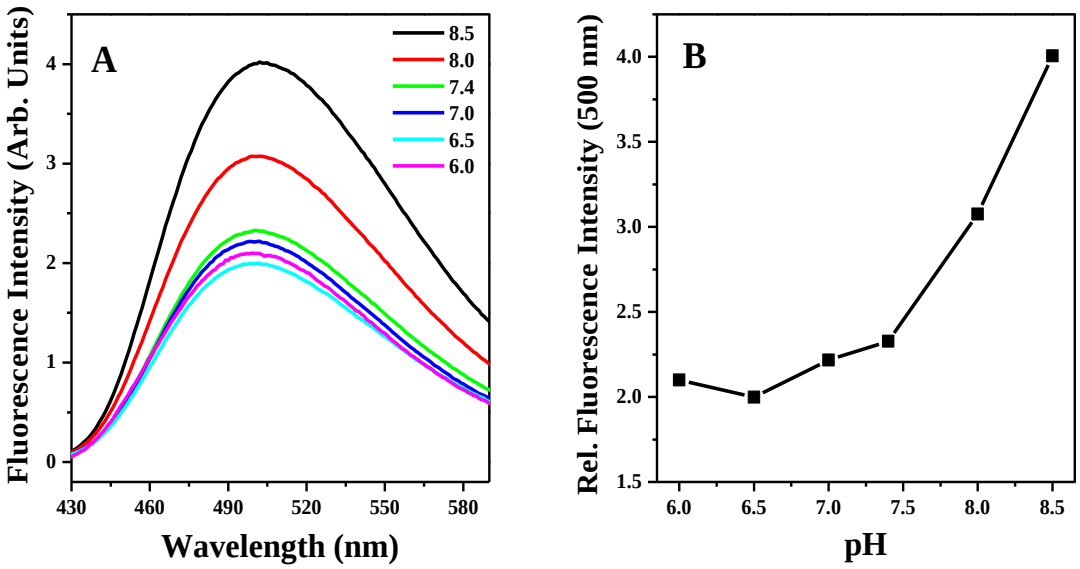


Fig. 5.9. Bis-ANS interaction with HSP-1/2. A) Difference fluorescence spectra of bis-ANS in the presence of HSP-1/2 at different pH. Each spectrum shown was obtained by subtracting the spectrum of bis-ANS alone from that of the bis-ANS/HSP-1/2 mixture. B) Relative fluorescence enhancement of bis-ANS in the presence of HSP-1/2 at different pH.

5.5. Summary

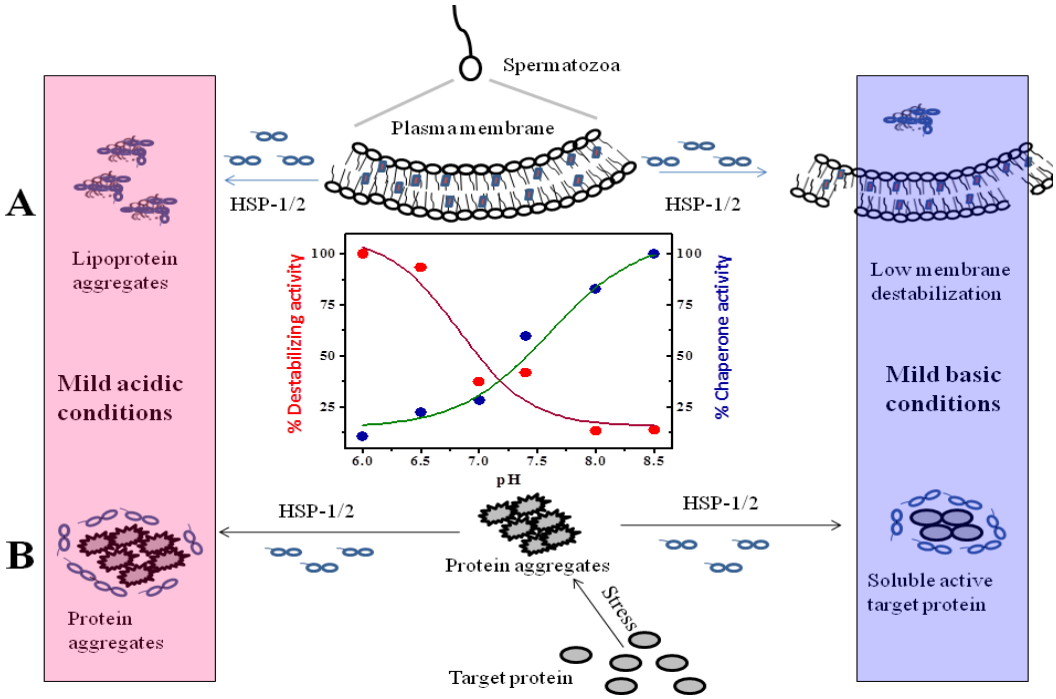


Fig. 5.10. Dual functionality of HSP-1/2 regulated by a pH switch. A) Cell membrane destabilization by HSP-1/2. B) Chaperone-like activity of HSP-1/2 towards substrate proteins

In the present work the effect of changing pH on the membrane destabilizing ability and CLA of HSP-1/2 were explored using various approaches. Erythrocyte lysis assays indicate that membrane destabilizing activity of HSP-1/2 is high at acidic pH and low at basic pH conditions. In contrast, the CLA of HSP-1/2 is very low at acidic pH and increases quite dramatically at basic pH. Our

results thus demonstrate that the membrane destabilizing and chaperone-like activities of HSP-1/2 exhibit an inverse relationship regulated by a pH switch as shown schematically in Fig. 5.10. To the best of our knowledge, this is the first study where two different activities/functions of a chaperone protein are regulated by a pH switch.

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General Discussion and Conclusions

Chapter 6

6.1. General discussion, conclusion and future prospects

This thesis presents results of studies carried out on various aspects related to the involvement of HSP-1/2 in sperm capacitation, fertilization and its possible role as a molecular chaperone in the reproductive tract. PDC-109, a analogous protein of HSP-1/2 from the bovine seminal plasma has been shown to be involved in *cholesterol efflux*, that is, release of cholesterol and phospholipids from the sperm plasma membrane thereby aiding in sperm capacitation [Desnoyers & Manjunath, 1992]. Recent studies have shown that PDC-109 also exhibits chaperone-like activity *in vitro*, by protecting various enzymes and proteins against stress conditions [Sankhala & Swamy, 2010]. This is a novel result because of the lack of conventional ATP dependent chaperone systems in the seminal plasma. This phenomenon shown by PDC-109, a FnII protein can be a species specific activity or a general activity among the seminal FnII proteins.

Chapter 2 addresses the above question and presents results which demonstrate that HSP-1/2 exhibits chaperone-like activity. HSP-1/2 is shown to protect heat induced aggregation of enzymes such as ADH, LDH, aldolase and carbonic anhydrase. Heat induced denaturation of G6PD resulted in loss of activity, whereas in the presence of HSP-1/2 the activity was regained to >90%, indicating protection offered by HSP-1/2. HSP-1/2 was also shown to protect insulin against aggregation induced by chemical stress. These results indicate that similar to PDC-109, HSP-1/2 also exhibits chaperone-like activity. Hence the chaperone-like activity can be a common phenomenon exhibited by the seminal FnII proteins. CD and computational modelling 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. It is also known that HSP-1/2 exists in monomeric form and as a ~90kDa polydisperse oligimer and that

binding of Phosphorylcholine (PrC) to it reduces the polydisperse nature of the protein to a predominantly monomeric form [Calvete et al., 1997]. PrC binding results in a decrease in the CLA of HSP-1/2, indicating that the polydisperse nature of the protein is important for its CLA. These results together with results previously obtained with PDC-109 [Sankhala & Swamy, 2010] indicate that polydispersity is an important factor for exhibiting CLA among seminal plasma FnII proteins [Sankhala et al., 2012].

Chapter 3 deals with chaperone like activity of HSP-1/2 under oxidative stress. Protection of target proteins by HSP-1/2 under heat and chemical stress conditions is relevant in sub-physiological conditions where proteins experience heat and chemical stress. Proteins of sperm and seminal plasma experience oxidative stress under normal conditions as reactive oxygen species (ROS) are produced in and by the spermatozoa during energy releasing pathways [Aitken et al., 1997]. This prompted us to investigate the CLA of HSP-1/2 under oxidative stress. The results of these investigations have shown that HSP-1/2 protects enzymes such as ADH and G6PDH against oxidative stress-induced loss of activity. It is also shown that HSP-1/2 prevents linoleic acid peroxidation, which indicates that HSP-1/2 possesses the ability to reduce/inhibit lipid peroxidation. This is of high physiological significance as equine spermatozoa contains high content of poly unsaturated fatty acids which in the presence of ROS produce lipid peroxides resulting in the loss of sperm structure and function [Agarwal et al., 2014]. Lipid peroxides were also found to be the major products in the spermatozoa stored in semen extenders; hence HSP-1/2 protection against lipid peroxidation is of great importance. HSP-1/2 was able to reduce the loss of fluorescence emission by fluorescein, a dye which in the presence of hydroxyl radicals loses its fluorescence. Further, some small heat shock proteins such as α-crystallin, which exhibits chaperone-like activity against oxidative stress towards

other proteins of eye lens, are susceptible to oxidation and it is shown that α-crystallin shows moderate loss of chaperone-like activity under oxidized conditions [Wang and Spector, 1995]. In this context, it is noteworthy that even under strong oxidizing conditions HSP-1/2 did not lose its chaperone-like and membranolytic activities. These results, together with the results presented in Chapter 2, indicate that HSP-1/2 protects biomolecules/proteins against heat, chemical and oxidative stress conditions.

Chapter 4 presents some new aspects and findings regarding interaction of choline phospholipids with HSP-1/2. Previous studies on interaction of choline phospholipids with HSP-1/2 have shown that similar to PDC-109, HSP-1/2 also specifically recognizes phospholipids containing the choline head group such as phosphatidylcholine and sphingomyelin. The interaction of HSP-1/2 with lipids perturbs the membrane structure by intercalating into the hydrophobic core of the membrane and results in the rigidification of the membrane [Greube et al., 2004]. Even though the interaction of HSP-1/2 with phospholipids observed is quite similar to that of PDC-109, quantitative differences are observed in the extent of immobilization of lipid membrane and extent of removal of phospholipids from erythrocyte membrane. Circular dicroism studies on the interaction of HSP-1/2 with PrC did not indicate any significant changes in the protein structure; hence to know more about the conformational changes associated with the ligand binding of HSP-1/2, we have employed fluorescence quenching studies on HSP-1/2 alone and in the presence of PrC, DVPC micelles and DMPC membrane. Upon interaction with the ligands, the degree of quenching has decreased with all the quenchers used: arylamide (neutral), cesium ion (cationic) and iodide ion (anionic). Binding of choline phospholipids afforded a protection to the tryptophan residues with the shielding order being: PrC ≤ divalaroyl PC < dimyristoyl PC. Similar to PDC-109,

HSP-1/2 has shown a REES both in the native state (3.5 nm) and after denaturation (3 nm), indicating water molecules are under motional restriction around tryptophan residues and that even after denaturation some of the structural and dynamic features of the microenvironment around the tryptophan residues are retained. HSP-1/2 exhibits only partial unfolding with chemical denaturants with no cooperativity, whereas complete and cooperative unfolding was observed in the presence of 10 mM dithiothreitol, indicating that disulphide linkages prevent complete unfolding of the protein. In the presence of PrC the transition midpoints shifted to higher concentrations of the denaturant together with a broadening of the sigmoidal transitions, indicating that ligand binding as well as polydispersity modulate the unfolding process.

Studies on the interaction of HSP-1/2 with model membranes using EPR and fluorescence spectroscopy have shown that HSP-1/2 induces membrane perturbation by intercalating into the hydrophobic core of the membrane, albeit to a lesser extent as compared to PDC-109 [Greube et al., 2004]. While HSP-1/2 has been reported to be less effective in lipid extraction compared to that of PDC-109, to date there have been no studies investigating the effect of HSP-1/2 into the structural integrity of the model membranes. The studies reported in ***Chapter 5*** give more insights on the effect of HSP-1/2 on the structural integrity of model membranes and various factors affecting it. AFM studies have shown that in the presence of HSP-1/2 DMPC did not form any membranous structure; rather, aggregates of ~80nm were observed which is very similar to earlier reports on PDC-109/DMPC interaction under similar experimental conditions [Damai et al., 2010]. HSP-1/2 was also found to destabilize erythrocyte cell membrane. Further, pre-incubation of HSP-1/2 with ligands such as PrC and choline chloride reduced the erythrocyte lysis, indicating that the mode of interaction between erythrocytes and HSP-1/2 is through its binding to choline

phospholipids. When the effect of pH on the chaperone-like activity was investigated, it was found that chaperone-like activity increases with increase in pH. The cell lytic activity is high at acidic pH and low at basic pH whereas the chaperone-like activity is high at basic pH but very low at acidic pH; that is, these two activities have an inverse relationship, regulated via a pH switch. This is a novel result, as to the best of our knowledge this is the first report demonstrating that dual functionality of a chaperone protein is regulated by a pH switch. The increase in the chaperone-like activity at basic pH could be correlated to an increase in the surface hydrophobicity of HSP-1/2, indicating that surface hydrophobicity is an important factor for chaperone-like activity of HSP-1/2.

The results presented in this thesis show that HSP-1/2 can act as a chaperone i*n vitro* and protect various proteins against heat, chemical and oxidative stress conditions. Studies on the interaction of choline phospholipids with HSP-1/2 have shown that the mode of interaction of HSP-1/2 is similar to that of PDC-109, but less effective in penetrating into the membrane core. Chaotrope induced unfolding studies have shown that HSP-1/2 resists chemical unfolding and that disulphide bonds, ligand binding and polydispersity modulate the unfolding process. Further, the membrane destabilizing ability of HSP-1/2 was probed and it was found that the membranolytic activity is high at acidic pH and low at basic pH whereas the chaperone-like activity is high at basic pH but very low at acidic pH, that is, these two activities have an inverse relationship, regulated via a pH switch. All these results will improve our understanding about the role of HSP-1/2 in the reproductive process and provide a detailed understanding of its chaperone-like activity. Such an understanding will be of considerable practical importance in veterinary and human medicine, e.g., for birth control and *in vitro* fertilization and in preparation and maintenance of semen extenders.

6.2. Future directions

This thesis gives details of chaperone-like activity of HSP-1/2, a major FnII protein of horse seminal plasma. Since FnII proteins are found in the seminal plasma of various mammalian and differ in structural and functional aspects with FnII proteins expressed elsewhere other than reproductive tract, it would be interesting to investigate whether FnII proteins of other species especially human exhibits chaperone-like activity, if so they can be targeted for therapeutics. As HSP-1/2 is found to inhibit lipid peroxidation, it would be of considerable interest to investigate the effect of addition of HSP-1/2 at basic pH to the semen extenders and verify whether it improves the sperm quality during storage. Further, interaction of HSP-1/2 with L-carnitine and acyl-carnitine can be interesting as L-carnitine is present in the equine seminal plasma and mimics choline group. As L-carnitine itself can act as an anti-oxidant, addition of HSP-1/2 complexed with L-carnitine can be useful in countering oxidative stress developed in semen extenders during long time storage.

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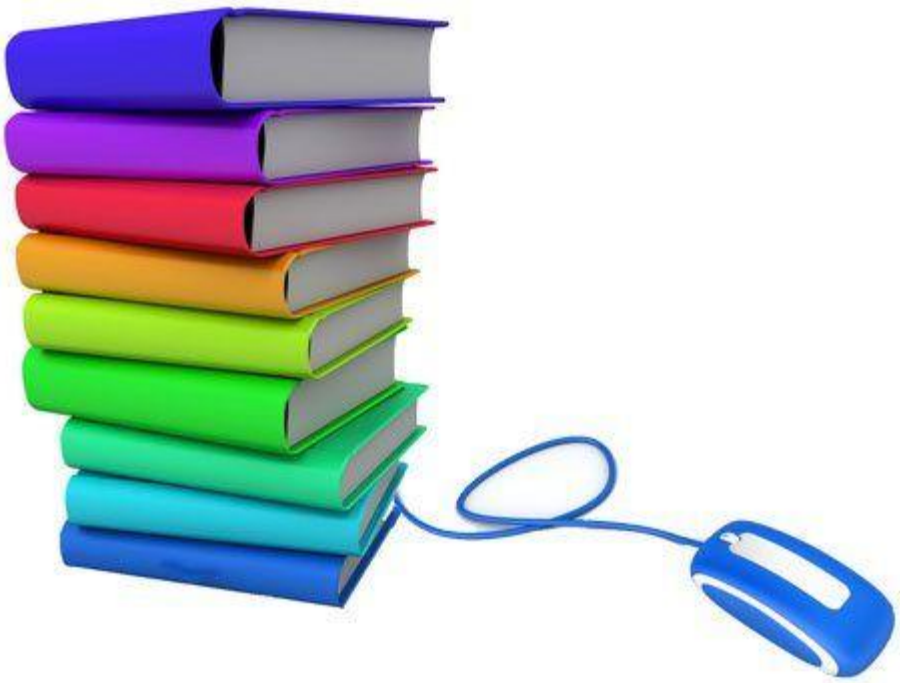

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Curriculum Vitae

Curriculam Vitae…

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C. Sudheer Kumar was born in Pulivendula, Kadapa {Dist.}, A.P. in 1988. He completed his primary education in Sri Bharghavi Vidyalaya, Muddanur. In Class VI, He got selected in Jawahar Navodaya Vidyalay (JNV) school entrance and carried out his schooling from class VI to XII at JNV, Kadapa (1998-2005). Then he moved to Loyola degree college, Pulivendula and obtained his B.Sc degree (Chemistry, Botony and Zoology) in 2008. He completed his Masters degree in Chemistry from University of Hyderabad in 2010. After clearing NET (JRF) in 2010 conducted by Council of Scientific and Industrial Research, India, he joined for Doctoral Studies (Ph. D.) under the supervision of Prof. M. J. Swamy, at School of Chemistry, University of Hyderabad in 2010. His major research focus is on "Molecular Biophysics, Structural Biology and Biochemistry and Biophysics of Protein interaction with other biomacromolecules", with the objective of understanding their structure-function relationships.

Curriculam Vitae…

List of Publications

1. Rajeshwar S. Sankhala, Rajani S. Damai, V. Anbazhagan, **C. Sudheer Kumar**, Gopalakrishna Bulusu and Musti J. Swamy. (2011) Biophysical investigations on Interaction of the major bovine seminal plasma protein, PDC-109 with heparin. *J. Phys. Chem. B* **115:** 12954–12962.

2. Rajeshwar S. Sankhala*, **C. Sudheer Kumar*,** Bhanu Pratap Singh, A. Arangsamy and Musti J. Swamy (2012) HSP-1/2, a major protein of equine seminal plasma exhibits chaperone like activity. *Biochem. Biophys, res. Commun.* **427:** 18-23.

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3. **C. Sudheer Kumar**, D. Sivaramakrishna, Sanjay K. Ravi and Musti J. Swamy. (2015) Fluorescence investigations on choline phospholipid binding and chemical unfolding of HSP-1/2, a major protein of horse seminal plasma. *J. Photochem. Photobiol. B: Biology* (Under Review)

4. **C. Sudheer Kumar** and Musti J. Swamy (2015) A pH switch regulates the inverse relationship between membranolytic and chaperone-like activities of HSP-1/2, a major protein of horse seminal plasma.(Under Review)

5. **C. Sudheer Kumar** and Musti J. Swamy. Chaperone like activity of the major horse seminal plasma protein, HSP-1/2 under oxidative stress. (To be Communicated)

6. **C. Sudheer Kumar** and Musti J. Swamy. Effect of surfactants on the chaperone like activities of HSP-1/2, a major protein of horse seminal plasma. (Manuscript under preparation).

7. **C. Sudheer Kumar** and Musti J. Swamy. Interaction of L-Carnitine with major protein of equine seminal plasma, HSP-1/2: Implication on membrane destabilizing and chaperone-like activities. (Manuscript under preparation).

Posters and Presentations:

1) Chaperone like activity of a major horse seminal plasma protein, HSP-1/2

Poster Presentation "REPROMIC-OMICS in reproduction and biology", Organized by Indian society of reproduction and fertility (ISSRF), February 7-9: 2012, Rajiv Gandhi centre for Biotechnology, Trivandrum, India.

2) Effect of surfactants on chaperone like activities of major horse seminal plasma protein, HSP-1/2

Poster Presentation 82nd Annual Meeting of the *Society of Biological Chemists* (India), "International Conference on Genome: Mechanism and Function", 2-5 December: 2013,held at University of Hyderabad, India.

3) Factors affecting chaperone like activity of major horse seminal plasma protein, HSP-1/2

Poster Presentation First Indo-Taiwan symposium on *Recent Trends in Chemical Sciences* (RCTS-2014), November 17-18, 2014, University of Hyderabad, Hyderabad .

4) Chaperone-like and lipid binding activities of a major protein of horse seminal plasma protein, HSP-1/2

Oral Presentation ChemFest-2015, 12th Annual In-House Symposium,

21st February, 2015, School of Chemistry, University of Hyderabad, Hyderabad, India.

5) Chaperone-like and Lipid binding activities of HSP-1/2, a major protein of horse seminal plasma.

Poster Presentation "DRILS Science Cafe-2015" , August 10th, 2015, Dr. Reddy's Institute of Life Sciences, University of Hyderabad, Hyderabad.