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HSP-1/2, a major horse seminal plasma protein, acts as a chaperone against oxidative stress

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ABSTRACT

The major protein of equine seminal plasma, HSP-1/2 exhibits chaperone-like activity and protects a variety of target proteins against thermal and chemical stress conditions. Here, we show that HSP-1/2 is able to protect enzymes such as alcohol dehydrogenase and glucose-6-phosphate dehydrogenase against H_2O_2 induced stress, clearly demonstrating that HSP-1/2 acts as a chaperone against oxidative stress. Further, the present studies show that HSP-1/2 also inhibits lipid (linoleic acid) peroxidation by hydroxyl radicals *in vitro*. These results are of great significance considering that so far limited or no antioxidative mechanism has been reported to be present in the mammalian spermatozoa that prevents lipid peroxidation which is detrimental to the motility and functioning of spermatozoa.

1. Introduction

The oxygen paradox is a necessary evil in most cellular systems. Cells under aerobic conditions constantly produce reactive oxidative species (ROS) and balance them through generation of antioxidants. When the regulation of ROS and antioxidants is imbalanced towards ROS, causing deleterious effects on lipids, proteins and DNA, the biological system is considered to be under “oxidative stress” [1]. Similar to many other cells, spermatozoa also produce ROS [2]. High concentration of ROS is observed in impaired sperm cells and is associated with loss of motility, decreased sperm–egg interaction and loss of fertility [3–5]. At low concentrations, ROS produced by spermatozoa are be involved in signaling processes controlling sperm capacitation and sperm–egg interaction [6–8]. This balance of ROS production in the semen is maintained by several factors present in the sperm and seminal plasma [9].

Seminal plasma, which carries spermatozoa from male testis to the female reproductive system also acts as a nutritional medium and protects spermatozoa from neutrophils in the female reproductive tract [10,11]. Enzymes glutathione peroxidase/reductase, superoxide dismutase and catalase along with several small molecules such as carnitine and taurine are responsible for the ROS scavenging capacity of seminal plasma [12].

HSP-1 and HSP-2 are major proteins of equine seminal plasma and account for more than 70% of its total protein content [13]. HSP-1 is a polypeptide of 121 amino acids with two fibronectin type-II (FnII) domains and an N-terminal flanking region with four glycosylation sites. The primary structure of HSP-2 is identical to that of HSP-1 but lacks a 14 amino acid segment at the N-terminus and is glycosylated to a lesser extent; their non-separable mixture is referred to as HSP-1/2 [14]. HSP-1/2 binds to a variety of ligands such as choline phospholipids, gelatin and heparin [15]. Among these, binding of HSP-1/2 to choline phospholipids has been investigated in detail as this interaction mediates its binding to sperm plasma membrane [16,17]. This interaction leads to *cholesterol efflux*, a key step in sperm 'capacitation', without which the sperm cannot fertilize the egg.

Previously, we have shown that HSP-1/2 and its bovine homolog PDC-109 exhibit chaperone-like activity (CLA) by protecting target proteins against thermal and chemical stress and that polydispersity and surface hydrophobicity are important for this activity [18–20]. These observations indicate that HSP-1/2 is able to protect other proteins against chemical and thermal stress conditions, *in vitro*. Results obtained in the present study show that HSP-

Abbreviations: ADH, alcohol dehydrogenase; ALD, aldolase; CLA, chaperone-like activity; DPPH, 2.2′- diphenylpicrylhydrazyl; EDTA, ethylenediamine tetraacetic acid; FnII, fibronectin type-II; G6P, glucose-6-phosphate; G6PD, glucose-6-phosphate dehydrogenase; HSP-1/2, horse seminal plasma proteins 1 and 2; NADP/NADPH, oxidized/reduced forms of nicotinamide adenine dinucleotide phosphate; PPC, p-aminophenyl phosphorylcholine; PrC, phosphorylcholine; PBS, 50 mM phosphate buffer containing 0.15 M NaCl, pH 7.4; PUFA, poly unsaturated fatty acids; ROS, reactive oxygen species.

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1/2 exhibits CLA under oxidative stress conditions as well by protecting target proteins and preventing lipid peroxidation by hydroxyl radicals.

2. Materials and methods

Materials and some methods are given in the Supporting Information.

2.1. Alcohol dehydrogenase activity under oxidative stress

Alcohol dehydrogenase (ADH) activity assay under oxidative stress was carried out as described in Ref. [21] with slight modification. The assay was initiated by adding 400 mM ethanol to the reaction mixture containing 0.5 μg/ml ADH and 2.5 mM NAD^+. Increase in absorption at 340 nm due to the formation of NADH was monitored using a Cary 100 UV–vis spectrophotometer (Agilent Technologies, Santa Clara, CA), which was used for all other spectrophotometric studies as well. Effect of oxidative stress on the ADH activity was assessed by pre-incubating the enzyme with 50 mM H_2O_2 for 10 min followed by activity measurement. To investigate the effect of HSP-1/2, ADH was pre-incubated with HSP-1/2 for 5 min before the addition of H_2O_2. Relative activities of various treated samples were normalized with respect to the activity of native enzyme. Experiments were done in triplicate and average values obtained are reported.

2.2. G6PD assay under oxidative conditions

G6PD activity was assayed spectrophotometrically as described earlier [19]. Aliquots from a 1 M stock solution of H_2O_2 were added to G6PD and incubated for 5 min before carrying out the assay. In another experiment G6PD was incubated with 10 mM H_2O_2 for different time periods before assaying for the activity. To investigate the effect of HSP-1/2 on the activity under oxidative stress, 0.5 μM of G6PD was incubated with 50 or 100 μg of HSP-1/2 before incubating with H_2O_2. Relative activities of various treated samples were normalized with respect to that of the native enzyme. All results reported are averages of three independent experiments.

2.3. Linoleic acid peroxidation assay

Linoleic acid peroxidation assay was performed as described earlier [22]. A 20 mM stock solution of linoleic acid was prepared in ethanol and ROS was generated from $FeSO_4$/ascorbate system. A 10 μl aliquot of the stock solution was added to give a final concentration of 0.2 mM linoleic acid to a solution containing 100 μM $FeSO_4$ and different concentrations of HSP-1/2. ROS were generated by the addition of ascorbate (10 μM final concentration). A control reaction was carried out at 37 °C in the absence of HSP-1/2. Formation of conjugated diene hydroperoxides due to oxidation was monitored by measuring absorbance at 234 nm. The protective activity of HSP-1/2 was defined as $[1-(A_{234}$ sample$)/(A_{234}$ control$)] \times 100$. All experiments were carried out at least twice and average values obtained have been reported.

2.4. Hydroxyl radical (•OH) detection assay using fluorescein

Hydroxyl radical detection was assayed using fluorescein as a probe [23]. Briefly, H_2O_2 was added to the reaction mixture containing 0.2 μM fluorescein to give a final H_2O_2 concentration of 50 mM. ROS was generated 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 fluorescence spectrometer (Champaign, IL) by exciting at 493 nm. The initial fluorescence intensities of fluorescein (after the addition of Co^{2+}) were normalized. The percent inhibition of hydroxyl radical was calculated using the formula $(100-(F_C/F_O) \times 100)$, where F_O and F_C are the normalized fluorescence intensities in the absence and in the presence of various additives at 60 min. All reported results are averages of at least two independent experiments.

2.5. Oxidation of HSP-1/2

HSP-1/2 was subjected to different oxidative conditions with respect to incubation time and concentration of oxidants used. In one set of samples (labeled HSP-1/2-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 h in PBS. In another set of samples (labeled HSP-1/2-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 h in PBS at room temperature. The reaction was stopped by adding 2 μl of catalase (10 U/μl) and after 5 min of incubation, the mixture was cooled on ice, dialyzed and stored at 4 °C. The effect of oxidation on chaperone-like activity and erythrocyte lysis activity of HSP-1/2 was probed as described earlier [19,24].

3. Results and discussion

3.1. Alcohol dehydrogenase activity under oxidative stress

Yeast ADH is a zinc containing tetrameric enzyme with multiple disulphide linkages. Under oxidative stress conditions ADH activity is lost due to conversion of Cys^{43} and Cys^{153} to Cys-SO_2H and Cys-SO_3H, respectively, with subsequent loss of zinc from the active site [21]. In order to investigate the ability of HSP-1/2 to protect target proteins against oxidative stress, activity of ADH in the presence of H_2O_2 was investigated. As shown in Fig. 1A (curve 3), ADH lost ~70% of the activity in the presence of H_2O_2 compared to that under native conditions (curve 1). In the presence of 10, 25 and 50 μg/ml of 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. Presence of HSP-1/2 under native conditions did not alter the activity of ADH (curve 2). A bar diagram representing percent activity of ADH under different conditions is shown in Fig. 1B.

3.2. G6PD activity under oxidative stress

G6PD, an important enzyme in pentose phosphate pathway, is involved in production of glutathione, which is the only oxidative stress regulating/inhibiting system in erythrocytes. The deficiency of G6PD leads to a condition called 'hemolytic anemia' [25]. G6PD 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 [26]. While carrying out experiments in the presence of Fe^{3+}/H_2O_2 system, we found that H_2O_2 alone was able to decrease the G6PD activity in a time and concentration dependent manner (Fig. 2A & B).

Further, effect of HSP-1/2 on the oxidative stress induced decrease in the activity of G6PD was investigated. G6PD shows ~40% residual activity under oxidative stress (Fig. 2C, curve 2). In the presence of 50 μg/ml of HSP-1/2, ~59% activity is retained and in the presence of 100 μg/ml of HSP-1/2, >90% activity is observed indicating the protecting ability of HSP-1/2 towards oxidative stress induced loss of G6PD activity. When the assay was carried out in the presence HSP-1/2 pre-incubated with G6PD without H_2O_2, a slight increase in the activity was observed, consistent with earlier observations [19]. Interestingly, another small heat shock protein