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Protein modification in the post-mating spermatophore of the signal crayfish *Pacifastacus leniusculus*: insight into the tyrosine phosphorylation in a non-motile spermatozoon

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ABSTRACT

After mating, spermatophores of signal crayfish are stored on the body of the female for a period before fertilization. This study compared the post-mating protein profile and pattern of protein tyrosine phosphorylation of the signal crayfish spermatophore to that of the freshly ejaculated spermatophore and found substantial differences. Two major bands of tyrosine-phosphorylated proteins of molecular weights 10 and 50 kDa were observed in the freshly ejaculated spermatophore of the signal crayfish. While the tyrosine-phosphorylated protein band with molecular weight 10 kDa was formed by protein(s) of similar pH, the band with molecular weight of 50 kDa consisted of proteins of varying pH. In the post-mating spermatophore, the band with molecular weight of 50 kDa was not detected, and an increase in the level of protein tyrosine phosphorylation was observed in the 10 kDa band. The microtubular radial arms of the spermatozoon showed a positive reaction to an anti-tyrosine antibody conjugated with gold particles in both the freshly ejaculated and post-mating spermatophores. In conclusion, the male gamete of the signal crayfish undergoes molecular modification during post-mating storage on the body of the female including changes in the level of protein expression and protein tyrosine phosphorylation. Structural similarity of the radial arms in the crayfish immotile spermatozoon with flagellum, which is the main site of protein tyrosine phosphorylation in the mammalian motile spermatozoa, raises questions regarding evolution and function of such organelles across the animal kingdom that must be addressed in the future studies.

1. Introduction

Phosphorylation of proteins refers to a post-translational modification event that acts as a cell regulatory mechanism to control various processes by addition and/or removal of phosphate groups from serine, threonine, or tyrosine residues of proteins that can induce allosteric modifications resulting in conformational changes in proteins leading to their activation or inactivation (Hunter, 2000; Naz and Rajesh, 2004; Pawson, 2004). Protein tyrosine phosphorylation in the spermatozoon occurs during sperm storage in the genital tract of the

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female, and is one of the most important modifications necessary for the mammalian spermatozoon to become functional for fertilization (Naz and Rajesh, 2004; Tulake et al., 2015; Kunkitti et al., 2015).

Post-mating sperm storage in the female reproductive tract occurs widely across all groups of vertebrate species such as reptiles, some fish, amphibians, all birds examined to date and mammals (Holt and Lloyd, 2010). Post-mating sperm storage has also been reported in decapods including shrimp (Wikramanayake et al., 1992; Alfaro et al., 2003, 2007; Vanichviriyakit et al., 2004; Aungsuchawan et al., 2011; Braga et al., 2014), crab (Hou et al., 2010), and crayfish (Dudenhausen and Talbot, 1983; López-Greco and Lo Nostro, 2008; Niksirat et al., 2014a, 2015b; Niksirat and Kouba, 2016). The spermatozoon of the shrimp *Sicyonia ingentis* undergoes a bi-phasic capacitation in the seminal receptacles of female (Wikramanayake et al., 1992). Experiments with shrimps indicated that further maturation of the spermatozoon is required in the female seminal receptacle (Alfaro et al., 2003). The further development of the spermatozoon filamentous meshwork during post-mating storage on the surface of the female was reported in shrimp *Litopenaeus vannamei* (Alfaro et al., 2007). Braga et al. (2014) reported morphological changes in the different parts of the pink shrimp *Farfantepenaeus paulensis* spermatozoon during capacitation.

Western immunoblotting has been employed to compare the level of protein tyrosine phosphorylation in freshly ejaculated and post-mating spermatozoa of *Penaeus monodon* (Vanichviriyakit et al., 2004). Aungsuchawan et al. (2011) used immunofluorescence microscopy to detect and compare the tyrosine-phosphorylated protein distribution pattern of freshly ejaculated spermatozoa with that of post-mating spermatozoa of the shrimp *Litopenaeus vannamei*. The acrosin activity of the spermatozoon of Chinese mitten crab *Eriocheir sinensis* is significantly higher in the spermatheca of mated females (Hou et al., 2010). New regulations of the proteins in the post-mating spermatophore of the noble crayfish *Astacus astacus* suggest that final maturation of male gamete takes place during storage on the female body (Niksirat et al., 2015b).

The crayfish spermatozoon lacks a true flagellum and is non-motile like other decapods (Tudge, 2009), making it a potential model for the study of morphological and molecular similarities to, and differences from, motile-type spermatozoa. The aim of the present study was to investigate possible changes in the level and subcellular localization of protein tyrosine phosphorylation in the signal crayfish *Pacifastacus leniusculus* spermatophore during post-mating storage on the body surface of the female.

2. Materials and methods

2.1. Animals

Male and female signal crayfish *Pacifastacus leniusculus* Dana, 1852 were collected from the Babačka Brook, Sklené nad Oslavou, Czech Republic in September 2013, transferred to the research facilities of University of South Bohemia, and kept in an outdoor pond under natural temperature and photoperiod. Pairs of signal crayfish including one male and one female were housed to facilitate natural mating. Electrical stimulation was used for extrusion of freshly ejaculated spermatophores from three specimens (Jerry, 2001). Samples of stored spermatophores were collected manually from the ventral surface of three females approximately 24 h after natural mating.

2.2. Two dimensional electrophoresis and Western blotting

2.2.1. Protein extraction and measurement

The sampled spermatophores were immersed in liquid nitrogen immediately after sampling and kept at −80 °C until analysis. Spermatophores were pulverized with a Cryopulverizer 5901-4H (Biospec Product Inc.) and mixed with cold 1% SDS. The samples were centrifuged at 16100*g* for 30 min. The protein concentrations of the supernatant were determined using Micro-Lowry Assay kit (Sigma-Aldrich).

2.2.2. Two-dimensional electrophoresis

One percent SDS was removed and replaced by DIGE lysis buffer (8 M Urea, 30 mM Tris, 5 mM Magnesium acetate, 4% CHAPS) using 0.5 mL ZebaTM Desalt Spin columns (Pierce). For fluorescent differential electrophoresis, proteins of each sample (freshly ejaculated and post-mating) were labelled by CyDye DIGE Fluor (Amersham Biosciences). One μl each of diluted CyDye 3 and 5 (200 pmol μL^{-1}) were added separately to 25 μg of protein of each sample and left on ice for 30 min in darkness. The reaction was stopped using 1 μL of 10 mM lysine and the sample was again left on ice for 10 min in darkness. An equal volume of sample buffer (8 M Urea, 0.02 g mL^{-1} DL-Dithiothreitol, 4% CHAPS, 2% IPG buffer) was added to the CyDye-labelled samples and left for 10 min on ice. Labelled samples were combined, and rehydration buffer (8 M Urea, 4% CHAPS, 0.25% IPG buffer, 2.8 mg mL^{-1} DL-Dithiothreitol) was added to the pooled samples to reach a final volume of 125 μL. The final solution was incubated for 30 min in darkness, centrifuged for 15 min at 16100*g*, loaded onto 7 cm strips (pH 3–10, BIO-RAD ReadyStrip™ IPG Strips), and overlaid with mineral oil (Plus one DryStrip Cover Fluid, Amersham Biosciences, UK). After passive rehydration for 14 h, isoelectric focusing (IEF) (Amersham Biosciences, Uppsala) was carried out for a total of 9600 Vh (30 min at 500 V, 1 h from 500 to 5000 V, 80 min at 5000 V). When IEF was completed, IPG strips were equilibrated in buffer I containing 6 M Urea, 50 mM Tris pH 8.8, 30% Glycerol, 2% SDS, 0.002% Bromophenol blue, and 10 mg mL^{-1} DL-Dithiothreitol for 15 min at room temperature under gentle agitation. A second equilibration was conducted under similar conditions in buffer II, which contained 25 mg mL^{-1} iodoacetamide instead of DL-Dithiothreitol. IPG strips were dipped into running buffer (0.25 M Tris, 1.92 M Glycine, 1% SDS) and embedded into 0.5% agarose sealing solution containing 0.002% Bromophenol blue on polymerized 12% SDS page (Bio-Rad, CriterionTGX). SDS page was run at a constant voltage of 200 V. The gel fixation was carried out by 30% ethanol and 10% acetic acid for 30 min and washed 3 times in water for 45 min. A Typhon