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A proteomic approach to identify seminal plasma proteins in roosters (*Gallus gallus domesticus*)

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ABSTRACT

Considering the interest in avian semen processing and storage, the objective of this study was to identify the domestic fowl seminal plasma proteins using two-dimensional gel electrophoresis (2-DE) and mass spectrometry MS/MS. For three times in a 4-month period, seminal plasma was obtained from semen collected from four local male chickens (*Gallus gallus domesticus*) and prepared for two-dimensional polyacrylamide gel electrophoresis. A total of 83 spots were detected across all gels and analyzed by MALDI-TOF/TOF. Among these spots, 17 have been successfully identified. The most intensely stained spots were recognized as serum albumin, ovotransferrin, alpha-enolase, fatty acid binding protein, thioredoxin, trypsin inhibitor CITI-1 and gallinacin-9. From these proteins, two are characteristic of avian seminal plasma, the ovotransferrin and gallinacin-9, and one is specific of the *Gallus* species, the chicken trypsin inhibitor CITI-1.

1. Introduction

In birds and in mammals, semen is a complex fluid comprising spermatozoa and seminal plasma (De Reviers, 1988; Fujihara, 1992; Killian et al., 1993; Lake, 1984; Solomon and Duncan, 2007). Seminal plasma contains many proteins that originate from the testis, epididymis (De Reviers, 1988; Fujihara, 1992; Lake, 1984) and in mammals also from the accessory glands (Eliasson, 1976; Mann, 1974). It provides a safe environment and allows spermatozoa to transit from the male to the female reproductive tract. Seminal plasma has also nourishing and buffering properties, absorbs end products of spermatozoa metabolism as well as influencing the structure and function of spermatozoa (Etches, 1996; Pilch and Mann, 2006). Sperm performance is not solely an attribute of the gamete, but is also determined by interactions with seminal plasma components, which are likely to have important effects on fertilization success (Al-Aghbari et al., 1992; Brandon et al., 1999; Fujihara, 1992; Rodriguez-Martinez et al., 2011; Slowinska et al., 2008; Yamakawa et al., 2007).

While there are many publications on the seminal plasma proteome of several mammals (Assumpcao et al., 2005; Brandon et al., 1999; Jobim et al., 2004, 2011; Kamaruddin et al., 2004; Mortarino et al., 1998; Novak et al., 2010; Pilch and Mann, 2006; Starita-Geribaldi et al., 2001) and insect (Baer et al., 2009; Reinhardt et al., 2009) species due to their veterinary, zootechnical, physiological and evolutionary interest, in avian species proteome studies have addressed muscle growth and development (Beynon and Pratt, 2005; Doherty et al., 2004; Hayter et al., 2005, 2003; McLean et al., 2004), hypothalamic markers (Kuo et al., 2005), craniofacial disorders (Mangum et al., 2005), ocular development (Lam et al., 2006; Wilmarth et al., 2004), blood plasma characterization (Corzo et al., 2004; Huang et al., 2006) and recently chicken sperm (Froman et al., 2011). Only a single pioneering, proteomic investigation has been conducted on rooster seminal plasma using

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disc electrophoresis to determine the number and concentration of proteins (Harris and Sweeney, 1971).

In birds, seminal plasma is of functional significance for spermatozoa as it contains sperm mobility stimulating factors (Ashizawa and Wishart, 1987; Blesbois and Caffin, 1992; Blesbois and De Reviers, 1992; Froman et al., 2006; Thomson and Wishart, 1989). Also among sexual functions of males there is adjustment of sperm quality and seminal fluid interactions based on extent of female attractiveness with dominant males increasing the velocity of sperm when ejaculation occurs with more attractive females through larger ejaculates that contain seminal fluid that increases sperm velocity (Cornwallis and O'Connor, 2009).

In contrast, when semen is stored before its use for artificial insemination, a deleterious effect of seminal plasma on spermatozoa has been observed for *in vitro* studies at low temperature (Blesbois and Caffin, 1992; Blesbois and De Reviers, 1992; Cornwallis and O'Connor, 2009; Douard et al., 2003; Thomson and Wishart, 1989). Blesbois and De Reviers (1992) found that low molecular weight seminal plasma fractions could reduce the fertilizing ability of spermatozoa during storage at 4 °C, whereas high molecular weight fractions appeared to enhance fertilizing ability. Douard et al. (2003), comparing the effects of turkey seminal plasma on spermatozoa stored at low and room temperature, suggested that the activation of phospholipase could explain the sperm degradation found at 4 °C when lipid peroxidation seems not to be active.

In a previous investigation, Castillo et al. (2011) detected for the first time ovotransferrin and gallinacin-9 in seminal plasma of roosters. Due to the antibacterial activity of these proteins (Michailidis and Avdi, 2010), ovotransferrin and gallinacin could have a role in protecting spermatozoa against microorganisms.

Considering the interest in avian semen processing and storage, it is surprising that up to now no proteomic investigation has been made to characterize the proteins of avian seminal plasma. This study uses two-dimensional gel electrophoresis and mass spectrometry to identify the seminal plasma proteins of the domestic fowl to achieve this objective.

2. Materials and methods

2.1. Chemicals

Immobilized PH Gradient (IPG) Strips, IPG buffer, acrylamide, molecular weight marker proteins were purchased from GE Healthcare (Uppsala, Sweden). Coomassie Brilliant Blue G-250 was purchased from AppliChem GmbH (Darmstadt, Germany). All other chemicals were purchased from Sigma (St. Louis, MO, USA).

2.2. Birds

Four male chickens from a local light breed (Brown Leghorn) belonging to the Experimental Avian Station of the Department of Veterinary Sciences of Pisa University were used to collect semen. Birds were 8-month-old fertile males as indicated by results from artificial inseminations. Males were housed individually in a 2.6 m^2 outdoor pen, under a natural increasing photoperiod (from 13L:11D to 15L:9D) and fed on a commercial breeder diet.

2.3. Semen and seminal plasma collection

Semen collections were performed three times in a 4-month period on the four birds by dorsal-abdominal massage. Care was taken to avoid contamination of semen with faecal matters by submitting roosters to an 8-h fasting time and care was also taken during the massage to avoid an excessive emission of the transparent fluid. Each day ejaculates were combined and seminal plasma was obtained by spinning pooled semen at 350 × *g* for 120 min in a Hermle Z300K centrifuge at 4 °C (speed 2000 rpm). The supernatant was recovered and immediately frozen in pellets by dropping 30 μL seminal plasma directly into liquid nitrogen. All seminal plasma pellets were stored at −196 °C until analyzed.

2.4. Sample preparation

Pellets from each day of collection were thawed. For each sample, 50 μL of seminal plasma was suspended in 100 μL of PBS pH 7.4 with 20 μL of Protease Inhibitor Cocktail solution (cod. P2714 Sigma–Aldrich) and lysed by applying four freeze–thaw cycles (Jousson et al., 2007). After centrifugation at 14,000 rpm for 20 min at 4 °C, the supernatant was recovered and the protein concentration was estimated by the Bradford assay using ovoalbumin as a reference. A volume of the supernatant containing 15 μg of protein was mixed with 10 drops of TCA 10% (w/v) and were placed in ice for 45 min before further centrifugation at 14,000 rpm for 20 min at 4 °C. The pellet was re-suspended in 160 μL of rehydration buffer containing 7 M urea, 2 M thiourea, 2% CHAPS, 60 mM DTT, 1% IPG buffer, and a trace of bromophenol blue. Seminal plasma protein (100 μg) was loaded on each 11 cm Immobiline Dry Strip pH = 3–10.

2.5. 2-DE (two-dimensional gel electrophoresis)

Isoelectric focusing electrophoresis was performed at 20 °C on a IPGphor III apparatus (Amersham Biosciences) following the same protocol reported in Dani et al. (2010, 2011) and Iovinella et al. (2011) modified as follows: the running schedule was 50 V for 1 h, 100 V for 1 h, 500 V for 1 h, 1000 V for 2 h, 3000 V for 4000 Vh in gradient, 4000 V for 3000 Vh in gradient, 6000 V for 3000 Vh in gradient, rehydration time 13.5 h, 50 μA for strip. Prior to SDS-PAGE the IPG strips were equilibrated in 6 M urea, 30% glycerol (v/v), 2% SDS (w/v), 1.5 M Tris–HCl, pH 8.8 and 1% DTT (w/v), for 15 min and afterwards in 6 M urea, 30% glycerol (v/v), 2% SDS (w/v), 1.5 M Tris–HCl, pH 8.8, 2.5% iodoacetoamide (w/v) and a trace of bromophenol blue for 15 min.

SDS-PAGE was performed using self-cast 12% T, 2.6% C separating polyacrylamide gels (1.5 mm thick) according to Laemmli (1970) but without stacking gel, using a vertical gel electrophoresis Hoefer SE 600 Ruby system (Amersham Biosciences). The run was conducted at 30 mA/gel at 8 °C for 3–4 h (Piccolomini et al., 2006).