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Identification and functional analysis of bull (*Bos taurus*) cauda epididymal fluid proteome

B. Westfalewicz,*[1] M. A. Dietrich,* A. Mostek,* A. Partyka,† W. Bielas,† W. Niżański,† and A. Ciereszko*
*Department of Gamete and Embryo Biology, Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, Tuwima 10, 10-748 Olsztyn, Poland
†Department of Reproduction and Clinic of Farm Animals, Faculty of Veterinary Medicine, Wrocław University of Environmental and Life Sciences, pl. Grunwaldzki 49, 50-366 Wrocław, Poland

ABSTRACT

Despite recent advances in bull epididymal fluid proteome research, significant numbers of proteins secreted to epididymal lumen remain unidentified. The objective of this study was to expand the number of identified cauda epididymal fluid proteins in bulls and to contextualize them in a broader view of their mutual interactions and involvement in biological processes and pathways, to fully elucidate the ways in which epididymal fluid proteins are involved in storage and maturation of spermatozoa in epididymis. We collected postmortem cauda epididymal fluid from 6 mature Holstein Friesian bulls. We performed the identification of proteins using 2-dimensional electrophoresis coupled with MALDI mass spectrometry. Analysis of functionality and pathway involvement of identified proteins was performed using Ingenuity Pathway Analysis software. We identified a total of 189 epididymal fluid proteins, out of which 100 were newly identified in bull epididymal fluid. We have combined our data with 2 previously performed bull epididymal fluid proteome identifications, yielding 280 proteins total, and analyzed it. The main canonical pathways involving epididymal proteins were glycolysis, gluconeogenesis, protein ubiquitination pathway, nuclear factor-erythroid 2-related factor 2-mediated oxidative stress response, and farnesoid X receptor/retinoid X receptor activation. The main biological functions potentially performed by epididymal fluid proteins included carbohydrate metabolism, cellular growth and proliferation, cell death and survival, and small molecule biochemistry. Overall, our results have pointed out multiple novel pathways in bull epididymal fluid that might take part in various aspects of maturation and protection processes of epididymal spermatozoa.

Key words: bull, epididymis, proteome

INTRODUCTION

The epididymis is a complex, convoluted tube that connects efferent ducts to the vas deferens in the male reproductive tract (Robaire et al., 2006; Dacheux et al., 2016). The mammalian epididymis usually consists of 3 regions: the initial segment, the head (or caput), the body (or corpus), and the tail (or cauda). The most basic function of the epididymis is the transportation of spermatozoa, delivering them from testicles to lower parts of the male reproductive tract. The epididymis also serves as effective storage for male gametes, preserving the viability of spermatozoa for up to 2 wk in the case of the bull. During transportation and storage, the absorptive and secretory activities of the epididymal epithelium create a specialized luminal environment that allows for sperm maturation. Epididymal sperm maturation is the final step of postgonadal sperm differentiation, during which spermatozoa acquire functional maturity manifested by development of fertilizing ability and motility. After ejaculation, mature sperm suspended in epididymal fluid is mixed with remaining secretions of male reproductive tract, in the case of the bull mainly seminal vesicle fluid, creating semen (Juyena and Stelletta, 2012).

Recent advances in electrophoresis, MS, and bioinformatics have allowed for a breakthrough in the comprehensive identification of mammalian semen proteins. Such an approach is called proteomics, which is research of the proteome (protein complement of the genome; Wilkins et al., 1996). Research of the bull reproductive tract proteome has led to the description of numerous proteins of seminal plasma and reproductive tract secretions (Kelly et al., 2006; Moura et al., 2007; Westfalewicz et al., 2017). This knowledge could be useful for cattle breeding for improving fertility or performance in sample storage (Soggiu et al., 2013; De Canio et al., 2014). Recently, we were able to describe the seminal vesicle fluid proteome and its functional similarity to seminal plasma proteome for the first time (Westfalewicz et al., 2017). Strong focus was directed

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[1]Corresponding author: b.westfalewicz@pan.olsztyn.pl

to find proteins connected with fertility localized in spermatozoa (D'Amours et al., 2010), seminal plasma (Odhiambo and Dailey, 2011), and epididymal fluid (Moura et al., 2006).

The first major work identifying bull epididymal secretion proteome was performed by Moura et al. (2010), who identified 30 proteins of cauda epididymal fluid. Belleannée et al. (2011) improved on this work and identified 172 luminal and secreted proteins from every region of the epididymis. Those works gave insight into the possible functionality of proteins identified and provided important information on changes occurring in the epididymal fluid of successive epididymal regions. Despite those advances, a significant part of the bull epididymal fluid proteome remains unexplored, as evidenced by several thousand proteins identified in epididymal secretions of more thoroughly investigated species such as humans or rats (Dacheux et al., 2016). Moreover, the description of proteins identified in bull epididymal fluid has been focused mainly on singular proteins and their evidenced or putative functions in the epididymis, with more comprehensive analysis being limited to rough functional classification of proteins using Gene Ontology tools. For this reason, we believe that further comprehensive research focused on mutual protein relationships, functions of protein groups, and protein involvement in metabolic pathways is justified.

In this study, we identified 100 new cauda epididymal fluid proteins and combined our results with those of previous epididymal fluid proteome identifications to obtain the most robust data set possible, consisting of 280 proteins. This combined data set was then analyzed for protein functions and metabolic pathways, to better understand bull epididymal fluid proteome functionality.

MATERIALS AND METHODS

Sample Collection

Samples were collected from six 4-yr-old Holstein Friesian bulls. The bulls were provided by the Breeding and Insemination Station in Karczew, Poland. Cauda epididymal fluid was collected postmortem from the bulls by the Department of Reproduction and Clinic of Farm Animals (Wrocław, Poland). The testicles of each bull were collected within 3 min after slaughter and transported at 4°C to the laboratory. In the laboratory, epididymides were dissected from the testes. The surface of each epididymis was thoroughly cleaned from the remnants of tissue and blood vessels and washed with physiological saline (0.9% NaCl). After removing the sodium chloride, the tail of the epididymis was punctured by an incision needle and the epididymal fluid containing spermatozoa was expelled by gentle pressure. Collection of the fluid was finished when the liquid changed color (from white to light pink). No additional fluids (buffers or PBS) were used for the liquid collection. Epididymal fluid was centrifuged for 30 min at 4°C (900 × *g*). Next, the supernatant was centrifuged for 10 min at 4°C (3,000 × *g*). After collection, the epididymal fluid was checked microscopically for the presence of blood cells. Samples with blood cells present were discarded. Supernatant was then stored at −80°C until the time of further studies.

Sample Preparation for 2-Dimensional SDS-PAGE

After thawing, samples of epididymal fluid (n = 6) were centrifuged for 60 min at 4°C (10,000 × *g*) to remove any remaining debris from the liquid. After centrifugation, aliquots containing approximately 700 μg of protein were cleaned with a Clean-Up Kit (GE Healthcare, Uppsala, Sweden) according to the manufacturer's protocol (https://www.gelifesciences.com/gehcls_images/GELS/Related%20Content/Files/1314735988470/litdoc80648964_20161013181035.pdf). The protein concentration before and after the cleaning procedure was measured by the Coomassie (Bradford) Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA).

2-Dimensional SDS-PAGE

Samples of cauda epididymal fluid containing 500 μg of protein were resuspended in rehydration buffer (7 *M* urea, 2 *M* thiourea, 2% {3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate}, 2% immobilized pH gradient buffer, 40 m*M* dithiothreitol, 0.002% bromophenol blue) to reach a final volume of 450 μL. Each sample was then loaded onto 24-cm Immobiline DryStrips, 3 to 10 nonlinear pH range (GE Healthcare), and rehydrated for 10 h. Proteins were then separated by isoelectric focusing on an Ettan IPGphor apparatus (GE Healthcare) operating at 20°C with current limited to 50 μA per strip and the following voltage program: 500 V/5 h, 1,000 V/1 h, 8,000 V/3 h, and 8,000 V/5.5 h. After isoelectric focusing, the strips were equilibrated for 15 min in SDS equilibration buffer (6 *M* urea, 75 m*M* Tris-HCl, pH 8.8, 29.3% glycerol, 2% SDS, and a trace of bromophenol blue) containing 10 mg/mL of dithiothreitol and then for 15 min in SDS equilibration buffer containing 25 mg/mL of iodoacetamide. The equilibrated strips were then transferred to manually cast 12.5% gels (25.5 × 19.6 cm, 1 mm thickness) and sealed with 0.5% agarose. A second dimension of electrophoresis was then performed at 1 W/gel in an Ettan Dalt-Six apparatus (GE Healthcare) for 16 h.