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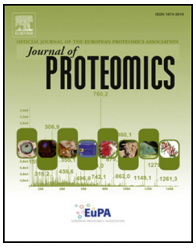

Analysis of epididymal sperm maturation by MALDI profiling and top-down mass spectrometry

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ABSTRACT

The fertilization ability of male gametes is achieved after their transit through the epididymis where important post-gonadal differentiation occurs in different cellular compartments. Most of these maturational modifications occur at the protein level. The epididymal sperm maturation process was investigated using the ICM-MS (Intact Cell MALDI-TOF MS) approach on boar spermatozoa isolated from four different epididymal regions (immature to mature stage). Differential and quantitative MALDI-TOF profiling for whole cells or sub-cellular fractions was combined with targeted top-down MS in order to identify endogenous biomolecules. Using this approach, 172 *m/z* peaks ranging between 2 and 20 kDa were found to be modified during maturation of sperm. Using top-down MS, 62 *m/z* were identified corresponding to peptidoforms/proteoforms with post-translational modifications (MS data are available via ProteomeXchange with identifier PXD001303). Many of the endogenous peptides were characterized as N-, C-terminal sequences or internal fragments of proteins presenting specific cleavages, suggesting the presence of sequential protease activities in the spermatozoa. This is the first time that such proteolytic activities could be evidenced for various sperm proteins through quantification of their proteolytic products. ICM-MS/top-down MS thus proved to be a valid approach for peptidome/degradome studies and provided new contributions to understanding of the maturation process of the male gamete involved in the development of male fertility.

Biological significance
This peptidomic study (i) characterized the peptidome of epididymal spermatozoa from boar (*Sus scrofa*); (ii) established characteristic molecular phenotypes distinguishing degrees

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of maturation of spermatozoa during epididymal transit, and (iii) revealed that protease activities were at the origin of numerous peptides from known and unknown proteins involved in sperm maturation and/or fertility processes.

1. Introduction

Mammalian spermatozoa acquire their fertilizing ability only after their transit through the epididymis, a single tubule several meters long [1]. Testicular spermatozoa are immobile and infertile. Their motility and their capacity to bind and penetrate the oocyte occur only in the epididymis. Such post-testicular sperm differentiation is linked to successive modifications of their protein and lipid [2–4] composition. During epididymal transit, the gametes progressively lose or modify most of their testicular surface proteins and gain new transient or permanent proteins in well-organized membrane protein domains [5–7]. These sequential modifications on the sperm membrane are associated with the sequential changes in the fluid which are believed to be directly or indirectly involved in sperm maturation during epididymal transit [8]. These changes result from various mechanisms, which include redistribution (or disappearance) after proteolytic processing, the action of glycolytic enzymes, and integration of newly synthesized components [9,10]. Numerous studies have been already developed to characterize and identify biomolecules potentially involved in the appearance and maintaining of fertility of the male gamete in the epididymis [7].

Most of these changes have been ascertained using classical proteomic approaches on intact or fractionated spermatozoa collected throughout the epididymis. The huge complexity of sequential surface changes related to epididymal maturation of mammal spermatozoa has been demonstrated by sperm surface labeling with either iodine or biotin and subsequent separation by SDS–PAGE [5]. Owing to the complexity of the sample preparation step using these techniques (i.e., sperm isolation, cell fractionation, optimization of one/two dimensional electrophoresis), these methods are time consuming and replicates limited, and most of these approaches cannot therefore be used for high-throughput screening.

Advances in mass spectrometry in combination with the development of bioinformatics tools during the past few years have allowed the development of global proteomic approaches based on profiling of intact cells and cell-free extracts [11]. MALDI-TOF MS has been used directly on isolated intact cells to obtain peptide and protein mass fingerprints in a mass range greater than 2,000 *m/z* or for cartography of lipids in a lower weight range (<1,000 *m/z*) [12–16]. This method, called Intact Cell MALDI-TOF-Mass Spectrometry (ICM-MS) or intact-cell or whole-cell mass spectrometry (ICMS or WCMS, respectively), is known for its applications in phyloproteomics on prokaryotes [17–23] and on eukaryotes such as yeasts [24]. MALDI-TOF MS-based identification has therefore been developed as a diagnostic tool readily available for routine, high-throughput analysis of bacteria and yeast isolates from clinical specimens [16,25]. This approach has been also extended for the identification of mammalian blood cells [26].

Differential analysis based on ICM-MS has also been used on prokaryotes to discriminate sensitive and resistant strains to antibiotics [27] and to characterize pathogenic biomarkers [28], to screen drugs *in vivo* and to quantify recombinant protein expression [29]. Comparative proteomic and peptidomic analyses have also been performed on different eukaryotic cells to detect apoptosis in cultured mammalian cells [30], to discriminate pancreatic islet alpha- and beta-cells [31] and neural cell subtypes [32], and to monitor cell differentiation [33] and viability and clone screening [34]. The differential MALDI MS-based approach has already been used on whole mammalian cells, and crude protein extracts and has shown that it can be a tool for easy and rapid access to the low mass range and constitutes a tool of choice for peptidomic studies to detect biomolecules in their functional and mature forms [35,36]. We recently explored the phenotype of chicken semen (intact spermatozoa and seminal plasma) with a focus on sub/infertile and fertile chickens, demonstrating that this sensitive method can be used as a new molecular tool for research on reproduction [37]. We therefore hypothesized that MALDI MS profiling combined with top-down MS would display and characterize unknown proteomic changes which occur in/on male boar (*Sus scrofa*) gametes during epididymal maturation processes.

The aim of our study was therefore (i) to compare characteristic MALDI profiles using distinguishing epididymal spermatozoa at different maturation stages by analysis of whole cells and enriching sperm sub-compartment extracts and (ii) to characterize and identify biomolecules potentially involved in the appearance and maintaining of fertility of the male gamete in the epididymis.

Whole cells were therefore analyzed by ICM-MS in order to characterize endogenous peptidoforms and proteoforms (<20 kDa) present in boar epididymal spermatozoa. Furthermore, in order to limit ion competition generally observed with complex sample analysis, we performed complementary experiments with detergent-soluble and detergent-insoluble spermatozoa extract fractions using MALDI-TOF MS. Moreover, to display sequential modifications potentially related to sperm maturation processes or fertility, MALDI profiling analyses were performed on the three preparations from cells isolated from 4 epididymal regions (immature to mature stages). Quantitative analyses and different statistical approaches (ANOVA, hierarchical clustering and PCA) were therefore applied to display the most significant differences between the maturation stages. A targeted top-down proteomic approach based on tandem high-resolution mass spectrometry was then performed on intact peptides and proteins to identify *m/z* peaks previously observed by MALDI profiling.

2. Materials and methods

2.1. Animals and organ sampling

Four 1-year-old adult boars (Large White) without known disease that could affect their fertility were used in this study.