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Acrosin inhibitor detection along the boar epididymis

Pavla Maňásková-Postlerová[a,*], Nina Cozlová[a,b], Andriy Dorosh[a], Miroslav Šulc[b,c], Benoit Guyonnet[d,e,1], Věra Jonáková[a]

[a] Laboratory of Reproductive Biology, Institute of Biotechnology, Academy of Sciences of the Czech Republic, v.v.i., Prague, Czech Republic
[b] Department of Biochemistry, Faculty of Science, Charles University, Prague, Czech Republic
[c] Institute of Microbiology, Academy of Sciences of the Czech Republic, v.v.i., Prague, Czech Republic
[d] INRA-CNRS-Universite de Tours-Haras Nationaux, Nouzilly, France
[e] Pôle Génétique, IFIP Institut du Porc, Le Rheu, France

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ABSTRACT

Epididymal sperm maturation represents a key step in the reproduction process. Spermatozoa are exposed to epididymal fluid components representing the natural environment essential for their post-testicular maturation. Changes in sperm membrane proteins are influenced by proteolytic, glycosylation and deglycosylation enzymes present in the epididymal fluid. Accordingly, the occurrence of inhibitors of these enzymes in the epididymis is very important for the regulation of sperm membrane protein processing. In the present study, we monitored acrosin inhibitor distribution in boar epididymal fluid and in spermatozoa from different segments of the organ. Using specific polyclonal antibody we registered increasing signal of the acrosin inhibitor (AI) from caput to cauda epididymis. Mass spectroscopy examination of the immunoprecipitated acrosin inhibitor (12 kDa) unequivocally identified sperm-associated acrosin inhibitor (SAAI) in the epididymal tissue. Lectin staining showed N-glycosylation in AI from boar epididymis. Protein detection of AI was supported by the results of semi-quantitative RT-PCR showing the presence of mRNA specifically coding for SAAI and similarly increasing throughout the epididymal duct, from its proximal to distal part. Additionally, the immunofluorescence technique showed the AI localization in the secretory tissue of caput, corpus and cauda epididymis, and in the acrosome region and midpiece of the sperm.

1. Introduction

During their passage through the epididymis, spermatozoa are directly exposed to the fluid containing various protein components (adhesion molecules, enzymes and their inhibitors, etc.) affecting post-testicular maturation of the sperm. Proteins present in the epididymal fluid have been described in many mammalian species including human [1]. Some of them bind to the surface of spermatozoa and may play a role in subsequent steps of the reproduction process, while others may affect sperm maturation. During the maturation, the protein components on the sperm surface are processed by enzymatic activity, mainly by proteases glycosyltransferases and glycosidases [2]. The balance between enzymes and their inhibitors seems to be necessary for maintaining a specific milieu in the testes and epididymis, and subsequently for the suitable gamete development. The main role of epididymal fluid proteases is to modify sperm surface proteins during epididymal maturation. One of the proteins processed enzymatically in the epididymis is angiotensin I converting enzyme (gACE) originating from the testes, which in caput epididymis is shed from the sperm surface [3]. Proteases are regulated by their inhibitors, which are richly represented among epididymal secretory products. One such protein is HE4 (Wfdc2), which is considered to be an epididymal tissue-specific extracellular proteinase inhibitor. It is a small cysteine-rich human protein secreted in the epididymis with two WAP domains, which belongs to the components of innate immune defense of epithelia [4]. Due to many cysteine residues in the molecule, protein HE4 could bind integral proteins of the sperm plasma membrane [5]. Another epididymal inhibitor is human serine proteinase cysteine-rich inhibitor EPPIN (SPINLW1). It contains the Kunitz domain consisting of three disulfide bonds and one WAP domain. EPPIN was localized on the sperm surface of capacitated and non-capacitated mouse sperm and in human seminal plasma, where it is bound to semenogelin I, one of the proteins abundant

* Corresponding author at: Institute of Biotechnology, Academy of Sciences of the Czech Republic, v.v.i., Vídeňská 1083, 142 20 Prague 4, Czech Republic.
E-mail address: pavlam@ibt.cas.cz (P. Maňásková-Postlerová).
[1] Present address: Evolution NT, 69 Rue De La Motte Brulon, 35700 Rennes, France.

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in human seminal plasma. The semenogelin-EPPIN complex exists either free in seminal plasma or bound to the sperm surface. EPPIN is thus a part of the sperm protective shell and its antibacterial activity protects sperm in the female reproductive tract and retains its viability [6]. Five more protease inhibitors have been identified in the mouse epididymis. Four of them (SPINK8, SPINK10, SPINK11, SPINK12) belong to the Kazal protease inhibitor family and the last one, WFDC10, contains the WAP domain. Gene expression of these proteins shows a very specific segmental pattern [7]. Acrosin is a major serine proteinase of mammalian sperm expressed in the testis [8], and is localized in the sperm acrosome [9,10]. The complex proacrosin/acrosin ensures specific sulphation of ZP glycoproteins, providing sufficiently long-lasting binding of acrosome-reacted sperm to ZP that facilitates the penetration process [11,12]. Proacrosin is highly expressed in all regions of the boar epididymis. In contrast, expression of α- and β-acrosin is low in the caput epididymis, but it increases along the organ and during *in vitro* capacitation. There is also redistribution of the proacrosin/acrosin complex at the apical ridge of the sperm head [13]. Boar acrosin inhibitor isolated from seminal plasma (SPAI) [14], also designated IACA, and sperm-associated acrosin inhibitor (SAAI) isolated from boar spermatozoa [15], also designated IACS, SPINK2, are structurally related. Their amino acid sequences are 90% identical [15] and both inhibitor isoforms are members of the Kazal-type subfamily [16]. The structure of SAAI was independently confirmed by the nucleotide sequence of its cDNA [17]. Glycosylation of SAAI with molecular mass of 8 kDa [15] has not been described, whereas SPAI is glycosylated and has a molecular mass of 12 kDa [14]. In our previous study, we detected acrosin inhibitor (AI) in almost all boar reproductive tissues and both isoforms of the acrosin inhibitor (8 and 12 kDa) on epididymal and ejaculated spermatozoa [18]. The physiological function of acrosin inhibitor is to protect sperm against the enzymatic activity of prematurely released acrosin, but it could also be one of the factors needed for stabilization of the binding sites on the sperm surface important for the egg-sperm interaction [19,20]. As proteolytic processing of the plasma membrane components during sperm epididymal maturation is required for the sperm development, the presence of inhibitors in epididymal fluid is necessary for the regulation of this event. In the present study we monitored boar epididymis and epididymal spermatozoa for gene and protein expression of acrosin inhibitors.

2. Materials and methods

2.1. Collection of biological fluids, spermatozoa and tissues from boar reproductive organs

Epididymides originated from boars (Large white boars) slaughtered at the Institute of Animal Physiology and Genetics (Liběchov, Czech Republic) and at INRA-CNRS (Nouzilly, France). Spermatozoa from the main parts of the epididymis (caput, corpus and cauda) were obtained by swimming up from the epididymal organ into phosphate-buffered saline (PBS) – 20 mM phosphate, 150 mM NaCl (pH 7.2) after incubation for 1 h at 37 °C. Spermatozoa were separated from the buffer by centrifugation (10 min at 600 × *g*) and were used for protein extraction and immunofluorescence.

Fluids with spermatozoa from the different epididymal zones 0-9 (caput 0/1-4; corpus 5-6; cauda 7-8/9) were microperfused as previously described by Dacheux [21]. Spermatozoa were separated from the fluid by centrifugation (10 min at 600 × *g* at 4 °C). The fluids were carefully removed and centrifuged again (10 min at 15,000 × *g*) and used directly or stored at −20 °C. Spermatozoa were washed three times with PBS, and then centrifuged for 15 min at 400 × *g*. Tissues of epididymal parts (0–9) and caput, corpus and cauda epididymis were cut into small pieces and kept at −70 °C to be used for the RNA and tissue protein extraction. Pieces of caput, corpus and cauda epididymis were frozen in blocks with tissue-freezing medium (Jung, Nussloch, Germany) in liquid nitrogen and stored at −70 °C. Cryosections of 3-μm thickness were prepared for the immunofluorescence technique.

2.2. Preparation of antiserum to seminal plasma acrosin inhibitor

Seminal plasma acrosin inhibitor was isolated as described in Davidová [18] by size exclusion chromatography on a Sephadex G-50 column followed by ion-exchange chromatography on a DE-cellulose 52 column. The fraction with inhibiting activity determined as amidase activity after hydrolysis of DL-BAPA (N-benzoyl-DL-arginine-4-nitroanilide hydrochloride) at 405 nm was purified by reverse-phase high-performance liquid chromatography (RP HPLC) in an HPLC system (LKB/Pharmacia, Uppsala, Sweden). Rabbit antiserum to the seminal plasma acrosin inhibitor (anti-SPAI) was prepared by immunizing female rabbits according to Davidová et al. [18]. The anti-SPAI antibody showed reactivity with both acrosin inhibitor isoforms – SPAI (12 kDa) and SAAI (8 kDa) [18].

2.3. Sperm and tissue extract preparation

Spermatozoa isolated from different parts of the epididymis (caput, corpus, cauda and epididymal zones 0/1-8/9) were extracted in 1% Triton X-100 for 30 min on ice. Epididymal tissues from caput, corpus and cauda (10 mg) were homogenized in 500 μl of 1% Triton X-100 in Tris–HCl (pH 7.8) with 50 mmol/l NaCl using homogenizer Precellys 24 (Bertin, Rockville, MD). Detergent was removed from the sperm and tissue extracts using 2-D Clean-Up Kit (GE Healthcare, Uppsala, Sweden) according to the manufacturer's protocol. Extracts were dissolved in non-reducing sample buffer (1% SDS in 0.5 M Tris–HCl, pH 6.8) for sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

2.4. Immunoprecipitation

Protein extracts (500 μg) of boar epididymis (caput, corpus and cauda) dissolved in 100 μl of 1% Triton X-100 in Tris–HCl (pH 7.8) with 50 mM NaCl were incubated with polyclonal serum anti-SPAI in 2:1 volume ratio (100 μl to 50 μl) for 2 h at 37 °C. Then, 50 μl of agarose-protein A/G beads (Sigma–Aldrich, St. Louis, MO) were added and incubated for 2 h at 37 °C. After centrifugation at 5000 × *g* for 10 min, protein A/G beads were washed tree times with 500 μl PBS and centrifuged at 5000 × *g* for 10 min. After washing, non-reducing sample buffer was added, beads were boiled for 5 min and then centrifuged at 5000 × *g* for 15 min. Supernatants were subjected to SDS-electrophoresis and acrosin inhibitor was detected with specific antibody on nitrocellulose membrane. The immunoprecipitated protein from epididymal tissue was subjected to MALDI-TOF MS analysis.

2.5. SDS-electrophoresis and Western blotting

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in 18% slab gel as described by Laemmli [22]. The protein samples (protein extracts from boar reproductive tissues in a concentration of 15 μg/μl) were dissolved in non-reducing buffer and boiled for 2.5 min at 100 °C. The molecular masses of the separated proteins were estimated using pre-stained precision protein standards All Blue from Bio-Rad (Hercules, CA) run in parallel.

Tris–glycine buffer (pH 9.6) with 20% methanol was used for the transfer of proteins separated by SDS-PAGE onto nitrocellulose membrane Hybond C-super (Amersham Biosciences, Uppsala, Sweden) for immunodetection. Electroblotting was carried out for

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