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Preliminary characterization of multiple hyaluronidase forms in boar reproductive tract

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Abstract

Hyaluronidases play an important role in gamete interaction and fertility in mammals. The objectives of the present study were to investigate multiple forms of the enzyme in boar reproductive tract using electrophoretic methods. Two forms of hyaluronidase (EC 3.2.1.35) were detected in boar seminal plasma (relative molecular masses of 55,000 and 65,000) using hyaluronic acid-substrate polyacrylamide gel electrophoresis in the presence of SDS. These two forms can be separated by means of affinity chromatography on Heparin-Sepharose. They differ, besides their affinity to heparin, also in the pH optimum of their enzymatic activity. The form with relative molecular mass of 55,000 was active both at the acidic (pH 3.7) and the neutral pH (pH 7.4) and was bound to immobilized heparin. The second form (relative molecular mass 65,000) was active only at acidic pH and did not interact with heparin. The same acidic-active form (65,000) was found in seminal vesicle fluids. The hyaluronidase form which is active both at the acidic and the neutral pH (51,000) was detected in epididymal fluid. In the detergent extracts of boar sperm, three active forms of the enzyme were found (relative molecular masses 55,000, 70,000 and 80,000). The form of relative molecular mass 55,000 was active in a wide range of pH (pH 3–8). The forms of relative molecular masses 70,000 and 80,000 were active only at neutral pH.

Keywords: Hyaluronidase; Boar sperm; Boar seminal plasma; Boar epididymis; Boar seminal vesicle

1. Introduction

Hyaluronic acid (HA), consisting of repeated disaccharide units of *N*-acetyl-D-glucosamine and D-glucuronic acid, is present in the extracellular matrix of different tissues. Hyaluronidase, enzyme hydrolyzing HA to oligosaccharides, is present in mammalian, insect and bacterial species. HA and hyaluronidase are implicated in many physiological processes including mammalian reproduction [1,2].

Two types of hyaluronidase were identified in the male reproductive organs: a glycosyl phosphatidyl (GPI)-anchored sperm-surface glycoprotein, and soluble forms. The sperm membrane-bound protein (PH-20) was initially characterized by Primakoff et al. [3] in guinea pig. This protein was later found on the sperm surface of many other species: mouse [4,5], cynomolgus macaque [6–8], human [9], stallion [10], rat [11], bull [12] and dog [13].

Sperm membrane-bound hyaluronidase (PH-20) is considered as a multifunctional protein involved in mammalian fertilization: (i) the enzyme activity is related to dispersion of cumulus oophorus by splitting the cumulus matrix formed by HA [4,6]; (ii) it might

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participate in the secondary binding of sperm to the zona pellucida following the acrosome reaction [4,7,14]; (iii) it may play a role in Ca^{2+} signaling-associated acrosomal exocytosis mediated by the hyaluronic acid receptor [15].

The sperm membrane-bound hyaluronidase forms were studied in detail in a number of species, as mentioned above. As far as characterization of soluble forms of this enzyme present in the male reproductive tract is concerned, less information is available and their role is not fully understood. Several authors have reported the presence of a soluble form of hyaluronidase, generated after the acrosome reaction, that may be involved in the penetration of zona pellucida and previtelline space by acrosome-reacted sperm [6,16–19].

Meyer et al. [12] showed the presence of a soluble form of hyaluronidase in bull testes and demonstrated that the 60,000 enzyme is a fragment of the PH-20 protein. Murine sperm PH-20 enzyme expressed in the epididymis is secreted in both a soluble and an insoluble form (epidimysomes) that contain an intact lipid anchor [20].

While the membrane-bound hyaluronidase (PH-20) of different species displays the enzyme activity at both neutral and acidic pH, the acidic activity is found in the soluble forms released after the acrosome reaction [6,9,21,22].

In comparison with other species, little information is available on hyaluronidases present in boar reproductive tract. Chondroitin sulfate A-derived oligosaccharides [23] and tannic acid [24] inhibited hyaluronidase activity extracted from boar sperm and effectively reduced polyspermy during in vitro fertilization of porcine oocytes. Porcine sperm adhesion molecule 1 gene (SPAM1) was characterized [25], as well as hyaluronidase cluster (SSC13q21) consisting of genes HYAL1, HYAL2 and HYAL3 [26]. Homologies of the porcine cDNA sequences to human orthologs were evaluated.

The objective of the present study is a basic characterization of multiple hyaluronidase isoforms present in boar seminal plasma, spermatozoa and in fluids of the male reproductive tract.

2. Materials and methods

2.1. Collection of reproductive organ fluids and spermatozoa

Fresh boar ejaculates were obtained from the insemination station Klimětice, Czech Republic. Ejaculates diluted in KORINATI medium (14.3 mM sodium bicarbonate, 12.25 mM sodium citrate, 364 mM glucose and 12.3 mM EDTA, pH 7.5) were centrifuged at 400 × *g* for 10 min to separate seminal plasma and spermatozoa. Spermatozoa were used for extraction of membrane proteins. Seminal plasma proteins were obtained by precipitation of boar seminal plasma with ammonium sulphate to 80% of saturation. Suspension was centrifuged (3000 × *g* for 20 min) and the precipitate was thoroughly dialyzed using Spectra/PorCE Membrane (MWCO = 2000) (Spectrum Medical Industries, Houston, TX) against distilled water and lyophilized.

Boar epididymis, seminal vesicle and prostate fluids were obtained from slaughtered boars, Institute of Animal Physiology and Genetics, Liběchov, Czech Republic. Epididymal ductus was impaled by injection and fluid was pushed out. Epididymal fluid and spermatozoa were separated by centrifugation for 15 min at 600 × *g*. Epididymal spermatozoa were washed twice with phosphate-buffered saline (PBS, 20 mM phosphate, 150 mM NaCl, pH 7.2) followed by centrifugation for 10 min at 600 × *g*. Epididymal fluid was frozen and stored at −20 °C.

The seminal vesicles and prostate gland separated from the connective tissue were cut away from the urethra and secretion was collected after applying pressure. After centrifugation (3500 × *g*, 15 min, 4 °C), the supernatant was frozen and stored at −20 °C.

2.2. Extraction of sperm membrane proteins

Spermatozoa were washed four times with PBS and then centrifuged for 10 min at 400 × *g*.

Sperm membrane-bound components were extracted by two different methods. First, the procedure described by Seaton et al. [11] was used. Briefly, washed sperm pellet (100 μl; ∼10^6 cells/ml) was incubated in PBS containing 1% Triton X-100 (Serva, Heidelberg, Germany) and 1 mM PMSF (phenylmethylsulfonyl fluoride, Sigma–Aldrich, St. Louis, USA) for 30 min at 4 °C. Sperm were centrifuged at 12,000 × *g* for 10 min at 4 °C.

SDS extract of sperm membranes was obtained by a modification of the procedure used in the case of cynomolgus macaque sperm [6]. Washed sperm suspension in PBS was centrifuged at 2000 × *g* for 5 min at 4 °C and supernatant was discarded. Sperm pellets were suspended in SDS extraction buffer (20 ml glycerol + 40 ml 10% SDS + 16 ml 1 M Tris–HCl buffer, pH 6.8 + 24 ml H_2O) and the sample was centrifuged at 2000 × *g* for 5 min at 4 °C.