Aggregated and Monomeric Forms of Proteins in Boar Seminal Plasma: Characterization and Binding Properties

(boar seminal plasma / carbohydrate, zona pellucida, phosphorylcholine and cholesterol-binding proteins / spermadhesins / sperm surface proteins and their aggregates)

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Abstract. Boar seminal plasma was separated into five protein fractions (I–V) (>100, 55, 45, 30, 5–15 kDa) by gel filtration chromatography on Sephadex G-75 SF at pH 7.4. RP HPLC of protein fractions I–V and N-terminal sequencing of their individual components revealed that high-molecular-weight aggregates consisted mainly of DQH sperm surface protein and AQN, AWN, PSP II spermadhesins, while fraction IV consisted of heterodimers of PSP spermadhesins only. Spermadhesins as monomers were present in seminal plasma in a very low amount. Biotinylated fractions I–IV containing AWN, AQN, DQH, and PSP proteins were bound to boar epididymal and ejaculated spermatozoa with the same efficiency. Aggregates containing AWN, AQN, DQH, PSP II proteins (fractions I–III) and their HPLC-separated monomeric forms interacted with phosphorylcholine. Aggregates containing the DQH protein and AWN spermadhesins as well as their separated monomeric proteins interacted strongly with acidic polysaccharides. PSP II interacted with some acidic polysaccharides, while the fraction IV corresponding to heterodimer PSP I/PSP II did not show any binding to acidic polysaccharides and zona pellucida. Fractions I–III showed affinity to cholesterol. The strongest interaction was observed between biotinylated glycoproteins of porcine zona pellucida and AWN 1-containing aggregates and separated proteins. AQN 1 spermadhesin efectivelly blocked the sperm binding to oocytes. These results suggest that under physiological conditions, the aggregated forms of seminal plasma proteins (DQH, AQN, AWN, PSP II) rather than the individual proteins might take part in coating the sperm surface, in sperm capacitation and in primary binding of spermatozoa to zona pellucida of the ovum.

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Abbreviations: ABTS – 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid), ACN – acetonitrile, BSA – bovine serum albumin, FITC – fluorescein isothiocyanate, PBS – phosphate-buffered saline, RP HPLC – reverse-phase high-performance liquid chromatography, SDS – sodium dodecylsulphate, TFA – trifluoroacetic acid.

Mammalian fertilization involves sperm capacitation, gamete recognition and binding of sperm to the ovum. All these events are mediated by specific interactions between molecules located on the surface of both gametes. Some proteins from seminal plasma that bind on the sperm surface upon ejaculation were shown to interact either with components of zona pellucida or with substances present in the natural environment of both gametes.

Individual proteins were isolated from seminal plasma of different species. Bull and boar seminal plasma proteins belong to the best characterized ones. Spermadhesins (AQN, AWN, PSP proteins, etc.) form a new family of low-molecular-mass (13–17 kDa) boar sperm surface proteins. They play a role in events leading to sperm capacitation, gamete recognition and binding to the ovum (Calvete et al., 1994). Spermadhesins also belong to the large family of developmentally regulated proteins with the characteristic CUB domain (Bork and Beckmann, 1993). The DQH sperm surface protein differs in its structure from boar spermadhesins. DQH homologous proteins have been detected in bull and stallion seminal plasma. The DQH protein is a member of the large family of cell and matrix adhesion proteins (Jonáková et al., 1998; Bezouška et al., 1999). Some physicochemical and binding properties of individual spermadhesins and DQH protein have been studied in detail earlier (Jonáková et al., 1991; Kwok et al., 1993; Sanz et al., 1993; Jonáková et al., 1995; Liberda et al., 1997; Tichá et al., 1998).

Interactions of seminal plasma proteins with the sperm surface and among themselves are supposed to play an important role in individual steps of the fertilization process. The formation of coating layers of spermadhesins on boar spermatozoa was studied by Dostálová et al. (1995a). Although the separated proteins from boar seminal plasma have been studied in detail, less attention has been paid to their physiological forms in seminal plasma. The binding properties of aggregated forms might differ from those of monomeric forms, as was already shown in the case of PSP proteins (Calvete et al., 1995). The formation of aggregates has already been described in some isolated sperm surface proteins (Calvete et al., 1997; Gasset et al., 1997). The heterodimer PSP I/PSP II

from boar seminal plasma (Calvete et al., 1995; Varela et al., 1997) represents a stable form of seminal plasma proteins.

It is shown here that under physiological conditions the boar sperm surface proteins preferentially exist as aggregates in seminal plasma. We focused on the characterization of these protein aggregates and studied their composition and binding properties.

Material and Methods

Material

Boar seminal plasma: boar semen was obtained from healthy Large White Pigs from the Insemination Station Nové Mlýny, Czech Republic. Boar seminal plasma was isolated from sperm ejaculate by centrifugation (600 *g*, 20 min, 5°C). Sperm isolation: ejaculates or epididymal fluid were centrifuged (300 *g*, 15 min, 20°C). The sperm pellet was resuspended in phosphate-buffered saline (PBS, pH 7.2) in a volume corresponding to removed seminal plasma or epididymal fluid and centrifuged under the same conditions. This step was repeated three times. Part of the pellet of ejaculated or epididymal sperm was then lyophilized. Capacitation of spermatozoa: one ml of fresh ejaculated sperm was diluted with 5 ml of sperm buffer (20 mM Hepes, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 95 mM NaCl, 1 mM D-glucose, 60 mM saccharose, 5 mM sodium pyruvate and 0.5% BSA, pH 7.4, 37°C). After 60 min incubation at 37°C, 1 ml of swim-up sperm was taken, diluted 1 : 1 with the same buffer and centrifuged at 180 *g* for 15 min. The pellet was resuspended in 10 ml of capacitation medium (1% medium TCM 199, 2.3% BSA, 26 mM $NaHCO_3$, 8 mM sodium lactate, 1 mM Hepes, pH 7.4) and incubated for 1 h at 37°C in a cell culture incubator (high humidity and 5% CO_2). For sperm-oocyte binding assays, the spermatozoa were diluted to 10^6 cells/ml.

Avidin, avidin-peroxidase, fluorescein isothiocyanate (FITC), N-hydroxysuccinimidobiotin, heparin, chondroitin sulphate, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), cholesterol and L-glycerylphosphorylcholine were purchased from Sigma, St. Louis, MO. Low-molecular-mass protein standards were the products of Serva, Heidelberg, Germany. Sephadex G-25, Sephadex G-75 Superfine (SF) were the products of Pharmacia Fine Chemicals, Uppsala, Sweden. Isolation of zona pellucida and its biotinylation was carried out according to Jonáková et al. (1998). Preparation of water-soluble polyacrylamide derivatives of acidic polysaccharides (heparin, chondroitin sulphate), their biotinylation and preparation of a fluorescein-labeled derivative of avidin (FITC-avidin) were described previously (Liberda et al., 1997). Biotinylated polyacrylamide derivatives of phosphorylcholine and cholesterol were prepared by conjugation of periodate-oxidized L-glyceryl-phosphorylcholine or maleinylated cholesterol to water-soluble polyacrylamide-allylamine copolymer (Liberda et al., 1999).

Gel chromatography on Sephadex G-75 SF

Boar seminal plasma (5 ml) was applied to a Sephadex G-75 SF column (2.0 × 118 cm) equilibrated with 0.1 M NH_4HCO_3, pH 7.4. Protein peaks eluted at the flow rate of 17.2 ml/h were pooled and lyophilized.

Reverse-phase high-performance liquid chromatography (RP HPLC)

Protein samples were subjected to inert Biocompatible Quaternary Gradient system of HPLC (Waters, Milford, MA). RP HPLC was performed using a 218 TP 54 Vydac C_{18} column (4.6 × 250 mm, 5, μm particle size). One mg of the sample in 1 ml of 0.05% trifluoroacetic acid was applied and proteins were eluted with a linear gradient of 20–50% acetonitrile in 60 min. Fractions corresponding to protein peaks were collected and lyophilized.

Molecular mass determination

The relative molecular mass of particular proteins was determined by gel chromatography on a Sephadex G-75 SF column (2.0 × 118 cm) using Blue Dextran, egg albumin (45 kDa), carbonic anhydrase (29 kDa), chymotrypsinogen A (25 kDa) and ribonuclease (14 kDa) as standards. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on 15% slab gels. The protein fractions I–V were dissolved in non-reducing buffer and were not boiled (Laemmli, 1970). Molecular mass of the separated proteins was estimated using protein standards run in parallel. Standards were prepared in reducing buffer (with 2-mercaptoethanol) and boiled 2.5 min at 100°C.

N-terminal amino acid sequence analyses

N-terminal amino acid sequences were determined automatically on the Applied Biosystems 470 A Protein Sequencer. The program O2R PTH was used and phenylthiohydantoin derivates of the amino acid residues were analysed by HPLC (Tsunasawa et al., 1985).

Biotinylation of proteins and their aggregates

N-hydroxysuccinimidobiotin (2 mg) dissolved in dimethyl formamide (50 μl) was added to the protein solution (2 mg) in 0.5 M $NaHCO_3$, pH 8.4 (4 ml). The solution was stirred for 30 min at 25°C. The reaction was stopped by addition of 1 M NH_4Cl (100 μl). The solution was dialyzed (MWCO = 2000) against PBS buffer, pH 7.2, overnight at 4°C. No significant changes in molecular masses of aggregates caused by biotinylation were detected by gel chromatography on a Sephadex G-75 SF column (1 × 65 cm).