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Biochemical and binding characteristics of boar epididymal fluid proteins

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ABSTRACT

During the passage through the epididymis, testicular spermatozoa are directly exposed to epididymal fluid and undergo maturation. Proteins and glycoproteins of epididymal fluid may be adsorbed on the sperm surface and participate in the sperm maturation process, potentially in sperm capacitation, gamete recognition, binding and fusion. In present study, we separated proteins from boar epididymal fluid and tested their binding abilities. Boar epididymal fluid proteins were separated by size exclusion chromatography and by high-performance liquid chromatography with reverse phase (RP HPLC). The protein fractions were characterized by SDS-electrophoresis and the electrophoretic separated proteins after transfer to nitrocellulose membranes were tested for the interaction with biotin-labeled ligands: glycoproteins of zona pellucida (ZP), hyaluronic acid and heparin. Simultaneously, changes in the interaction of epididymal spermatozoa with biotin-labeled ligands after pre-incubation with epididymal fluid fractions were studied on microtiter plates by the ELBA (enzyme-linked binding assay) test. The affinity of some low-molecular-mass epididymal proteins (12–17 kDa and 23 kDa) to heparin and hyaluronic acid suggests their binding ability to oviductal proteoglycans of the porcine oviduct and a possible role during sperm capacitation. Epididymal proteins of 12–18 kDa interacted with ZP glycoproteins. One of them was identified as Crisp3-like protein. The method using microtiter plates showed the ability of epididymal fluid fractions to change the interaction of the epididymal sperm surface with biotin-labeled ligands (ZP glycoproteins, hyaluronic acid and heparin). These findings indicate that some epididymal fluid proteins are bound to the sperm surface during epididymal maturation and might play a role in the sperm capacitation or the sperm–zona pellucida binding.

1. Introduction

Mammalian spermatozoa leaving the testis do not have the ability to interact with oocyte and fertilize it [1]. They gain these abilities while passing through the epididymis. The epididymis is a tissue with very active fluid-absorbing and fluid-secreting activities and the fluid contains ions, amino acids, small organic molecules, proteins, glycoproteins and a wide range of enzymes (for review see [2–4]). Because there are no known synthetic activities of spermatozoa from the testis, the changes in sperm surface are thought to be due to their interaction with the luminal fluid within the epididymis. The surface of spermatozoa is exposed directly to the epididymal fluid, and sperm plasma membrane undergoes significant changes during the passage. This process is called epididymal (post-testicular) maturation and covers changes in distribution of intramembraneous proteins or glycoproteins of testis origin, but some testicular proteins are also altered, masked, or replaced by proteins of epididymal origin [1,5–8]. As a result of these modifications, the spermatozoa become motile and functionally competent cells capable of undergoing capacitation and binding to the zona pellucida, the extracellular coat surrounding the mammalian oocyte. Epididymal proteins associated with the sperm surface during maturation are thought to have a particular role in further steps leading to fertilization. However, the exact role of most of them is still unclear. Epididymal secretion is sequential mainly due to the spatial restrictions of the organ and specific protein reabsorption. It means that the fluid composition changes continuously along the tubule [9]. Several proteins produced by the epididymis have been described in various mammalian species: rat, mouse, hamster, boar, ram, stallion, and human. Major mam-

Abbreviations: ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt; Crisp, cysteine-rich secretory protein; CTP, cholesterol transfer protein; ELBA, enzyme-linked binding assay; GPX5, glutathione peroxidase 5; HE, human epididymal proteins; Hep$^+$, heparin-binding fraction; Hep$^-$, non-heparin-binding fraction; PBS, phosphate-buffered saline; PGDS, prostaglandin D2 synthase; RP HPLC, high-performance liquid chromatography with reverse phase; SDS-PAGE, sodium dodecylsulphate-polyacrylamide gel electrophoresis; TMB, 3,3′,5,5′-tetramethylbenzidine; ZP, zona pellucida.

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malian epididymal secretory proteins are clusterin, prostaglandin D2 synthase (PGDS), glutathione peroxidase 5 (GPX5), lactoferrin, cholesterol transfer protein (CTP) and glucosidases [8,10].

Six human epididymal cDNA families, named HE1–HE6, were identified by Kirchhoff [11,12] and Kirchhoff et al. [13]. The predicted proteins have been localized in the human epididymal epithelium, within the lumen of the epididymal duct and vas deferens, and also on the surface of ejaculated spermatozoa [11]. Boar epididymis also produces closely related counterparts to human epididymal proteins HE1, HE3, HE4, HE5 and HE12 [14].

In human, mouse and rat, epididymal proteins belonging to the Crisp (cysteine-rich secretory protein) family have been described [15]. Rat epididymal DE (Crisp1) protein is localized in the equatorial segment of the sperm head and plays a specific role in fertilization, in sperm–egg fusion through its binding to egg-complementary sites [16].

The sperm plasma membrane undergoes extensive changes not only during the epididymal maturation, but also during ejaculation and sperm capacitation in the oviduct. In all these events the sperm surface is exposed to components secreted both by the male and female reproductive tracts (epididymal fluid, seminal plasma and oviductal fluid). Proteins secreted by epididymis and accessory sex glands can bind the sperm surface and might play a role as a protective layer against the relatively hostile environment in the female reproductive tract. Many proteins of seminal plasma serve as decapacitating factors and during the sperm capacitation process they leave the sperm surface [17]. Such protein dissociation is supported by proteoglycan molecules present in oviductal fluid, especially glycosaminoglycans of heparin and chondroitinsulphate types [18]. A positive effect of hyaluronan on the sperm capacitation in pig has also been described [19].

In the present study, we characterized proteins of fluid isolated from boar cauda epididymis. Epididymal proteins were separated using various chromatographic and electrophoretic methods. Separated proteins were tested for their ability to interact with model and endogenous biotin-labeled ligands (ZP glycoproteins, hyaluronic acid and heparin) suggesting their prospective roles in the individual steps of reproduction. Some of the epididymal proteins with ligand-binding activity were identified.

2. Materials and methods

2.1. Collection of boar epididymal fluid and epididymal spermatozoa

Boar epididymal fluid together with epididymal spermatozoa was obtained from epididymis of slaughtered boars (5 adult animals were used for these experiments). Epididymal ductus was impaled by injection and fluid was pushed out. Epididymal fluid was centrifuged for 15 min at 600 × *g*. Spermatozoa were thoroughly washed five times with phosphate-buffered saline (PBS – 20 mM phosphate, 150 mM NaCl, pH 7.2), followed by centrifugation for 10 min at 600 × *g*.

2.2. Preparation of solubilized zona pellucida

Porcine ovaries (20 pieces) were obtained from slaughtered adult sows from slaughterhouse Písnice (Prague, Czech Republic). Oocytes were released from frozen porcine ovaries in a meat grinder with ice-cold saline (0.15 M NaCl) and homogenate was sieved through nylon screens as described by Hedrick and Wardrip [20]. The oocytes were purified by centrifugation in a discontinuous Percoll gradient [21]. The oocytes were collected from 0 to 10% interface, washed in distilled water and gently homogenized using small glass homogenizer. The zona pellucida particles were collected on a 40 μm screen and repeatedly washed with saline. Isolated zonae pellucidae were heat solubilized in 0.2 M $NaHCO_3$, pH 9 at 73 °C for 30 min and centrifuged at 350 × *g* for 10 min. The supernatant was used for biotinylation [22].

2.3. Biotinylation of ligands and zona pellucida glycoproteins

The preparation of water-soluble poly(acrylamide-allylamine) derivatives of heparin and hyaluronic acid and their biotinylation have been described previously [23,24]. Solubilized zona pellucida (in protein concentration 0.160 mg/ml) was incubated with 0.4% (m/v) *N*-hydroxysuccinimidobiotin (Sigma–Aldrich, St. Louis, MO) in dimethylformamide (Sigma–Aldrich, St. Louis, MO) for 30 min at room temperature [22]. The biotinylated ligands and zona pellucida glycoproteins were dialyzed in Spectra/Por MWCO 6/8000 membrane against PBS overnight and stored at −20 °C.

2.4. Size exclusion chromatography on Sephadex G-75 M

Boar epididymal fluid (5 ml) diluted 1:1 with PBS, pH 7.2 was applied to a Sephadex G-75 Medium (Pharmacia Fine Chemicals, Uppsala, Sweden) column (2.0 cm × 115.0 cm) equilibrated with 1.5% (v/v) acetic acid, pH 3.0. Protein peaks eluted at the flow rate of 17.8 ml/h were pooled and lyophilized.

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

Protein fractions obtained by size exclusion chromatography (EF1–3) were subjected to inert Biocompatible Quaternary Gradient system of HPLC (Waters, Milford, MA). RP HPLC was performed using a 218 TP 104 Vydac C_{18} column (4.6 mm × 250 mm, 10 μm particle size). One milligram of the sample in 1 ml 0.05% (v/v) trifluoroacetic acid (Merck, Darmstadt, Germany) was applied and proteins were eluted with a linear gradient of acetonitrile (Merck, Darmstadt, Germany). Fractions corresponding to protein peaks were collected and characterized by SDS-PAGE.

2.6. Affinity chromatography of epididymal fluid fractions on immobilized heparin

Affinity chromatography of epididymal fluid fractions EF1–3 on immobilized heparin was performed according to the protocol of boar seminal plasma separation as described previously by Jonáková et al. [22]. Briefly, epididymal fluid fractions (10 mg dissolved in 10 ml of PBS) were applied to a heparin-Sepharose CL 6B (Amersham, Uppsala, Sweden) column (2.8 cm × 5.5 cm) and washed in PBS. Non-adsorbed proteins (Hep^-) were eluted with the starting buffer and 3 M NaCl was used to elute adsorbed proteins (Hep^+). Heparin-binding and non-binding fractions were characterized by SDS-PAGE.

2.7. SDS-electrophoresis and Western blotting

Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on 15% slab gels [25]. Polyacrylamide gel (15%T) was prepared with 29.2% (m/v) acrylamide and 0.8% (m/v) bisacrylamide in 1.5 M Tris buffer (titrated by HCl to pH 8) and with 0.1% (m/v) sodium dodecylsulphate (SDS). The stacking gel (4% T) was composed of acrylamide/bisacrylamide in 0.5 M Tris buffer (titrated by HCl to pH 6.8) and 0.1% (m/v) SDS. Electrophoretic separation was carried out in Tris–glycine electrophoretic buffer, pH 8.3 (25 mM Tris, 192 mM glycine) with 0.1% (m/v) SDS at constant voltage of 80 V for 30 min and of 145 V for 1 h at room temperature. The protein samples 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 prestained Precision Plus Protein Standards from Bio-Rad (Hercules, CA) run in parallel.

Tris–glycine buffer, pH 9.6 (25 mM Tris and 192 mM glycine) with 20% (v/v) methanol was used for transfer of proteins separated by SDS-PAGE onto Hybond C-super nitrocellulose membrane (Amersham, Vienna, Austria) for the binding assays (Far Western blot) or Immobilon-P-membrane (Serva, Heidelberg, Germany) for N-terminal amino acid sequence analysis. Electroblotting was carried out for 1.5 h at 500 mA at 6 °C and according to the arrangement as described by Towbin et al. [26].

2.8. Far Western blot test with biotin-labeled ligands

The nitrocellulose membrane with the transferred proteins was deactivated with 0.5% (m/v) Teleostean fish gelatin (Sigma–Aldrich, St. Louis, MO) in PBS at 4 °C overnight. After washing with 0.02% (v/v) Tween 20 (Serva, Heidelberg, Germany) in PBS, the membrane was incubated with biotin-labeled ligands (polyacrylamide derivatives of heparin and hyaluronic acid – 100 μg/ml in PBS, glycoproteins of porcine zona pellucida (gpZP) – 10 μg/ml in PBS) for 2 h. Following washing, the incubation with avidin-peroxidase (Sigma–Aldrich, St. Louis, MO) solution of 0.25 μg/ml in 0.1 M Tris buffer titrated by HCl to pH 8.0 was performed for 1 h. After washing, the membrane was developed with TMB (3,3′,5,5′-tetramethylbenzidine) solution (Sigma–Aldrich, St. Louis, MO). The reaction was stopped after 10 min by washing the membrane in distilled water. All procedures were carried out at room temperature.

2.9. N-terminal amino acid sequence analysis

Proteins transferred onto the Immobilon-P-membrane were used for the N-terminal amino acid sequencing. The N-terminal amino acid sequence was determined by Edman degradation, formed phenylthiohydantoin derivatives were analyzed by HPLC. Protein sequencing was performed automatically in the Applied Biosystems 470 A Protein Sequencer (Foster City, CA). Protein sequences were determined using the BLAST database (http://blast.ncbi.nlm.nih.gov).

2.10. Enzyme-linked binding assay (ELBA) – binding of biotin-labeled ligands to epididymal spermatozoa with a contribution of epididymal fluid proteins

Wells of a microtiter plate were coated with fresh epididymal spermatozoa (dilution 1:20 in PBS, 100 μl) and the plate was incubated overnight at 4 °C. After washing with PBS containing 0.05% (v/v) Tween 20, the wells were deactivated with 200 μl of 0.5% (m/v) Teleostean fish gelatin (Sigma–Aldrich, St. Louis, MO) in PBS for 2 h at room temperature. After washing, 100 μl epididymal fluid fractions (EF1–3 – 100 μg/ml PBS) were added to some wells, and the plate was incubated for 4 h at 37 °C. After washing and addition of 100 μl solution of biotin-labeled ligands (heparin, hyaluronic acid or gpZP – dilution range from 100 to 12 μg/ml in PBS) to all wells, the plate was incubated overnight at 37 °C. Following washing, the incubation with 100 μl per well avidin-peroxidase solution (0.25 μg/ml in 0.1 M Tris buffer titrated by HCl to pH 8.0) was performed for 1 h at room temperature. The plate was washed again, and the enzyme reaction was initiated by adding 100 μl of 0.01% (m/v) 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) (Sigma–Aldrich, St. Louis, MO) in 0.1 M phosphate–citrate buffer (pH 4.6) with 0.09% (v/v) hydrogen peroxide to each well. After 10 min incubation in the dark, absorbance at 405 nm was measured using a microplate reader (SLT-Spectra, SLT-Labinstruments, Vienna, Austria). The measurement of all samples was carried out in triplicate with insignificant deviation of the resulting values.

3. Results

3.1. Separation and characterization of epididymal fluid proteins

By size exclusion chromatography, boar epididymal fluid was separated into three protein fractions (EF1–3) according to the molecular masses (Fig. 1A). Separated protein EF1–3 fractions were characterized by SDS-PAGE under non-reducing conditions (Fig. 1B). Fraction EF1 contained mainly high-molecular-mass proteins of 30–150 kDa. Fraction EF2 consisted of proteins in a wide range of molecular masses 12–150 kDa and fraction EF3 of proteins of 8–25 kDa.

3.2. Fractionation of epididymal fluid proteins by RP HPLC

Protein fractions after size exclusion chromatography (EF1–3) were subjected to high-performance liquid chromatography with a reverse phase column (RP HPLC). Each EF fraction was separated into 10–14 subsequent protein fractions: EF1 (1_1–10_1), EF2 (1_2–14_2) and EF3 (1_3–14_3). Chromatograms in Fig. 2A–C represent the particular separation of EF1–3 fractions in different acetonitrile gradients optimized for each protein EF fraction. HPLC-separated fractions were characterized by SDS-PAGE (Fig. 3A–C). After HPLC separation, other protein bands with low molecular masses were found in epididymal fluid fractions (EF1–3) non-visible after size

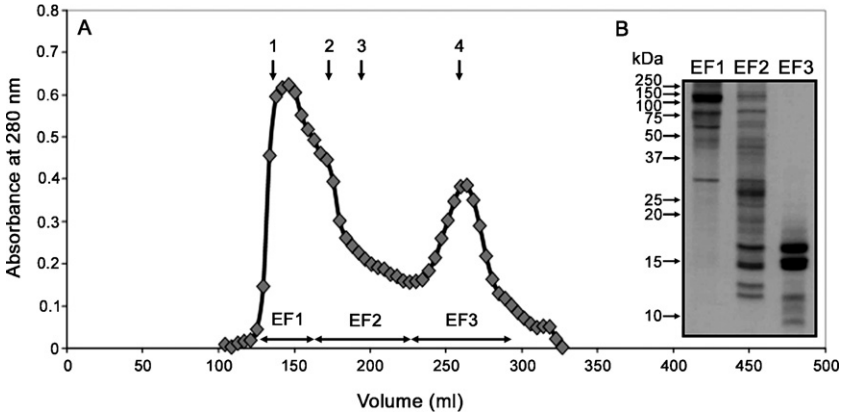

Fig. 1. (A) Size exclusion chromatography of boar epididymal fluid: separation into three protein fractions according to molecular mass (EF1–3); molecular mass protein markers: 1 – >75 kDa (Blue Dextran), 2 – 45 kDa (egg albumin), 3 – 29 kDa (carbonic anhydrase), 4 – 13 kDa (ribonuclease); (B) separation of protein fractions EF1–3 after size exclusion chromatography by 15% SDS-PAGE under non-reducing conditions.

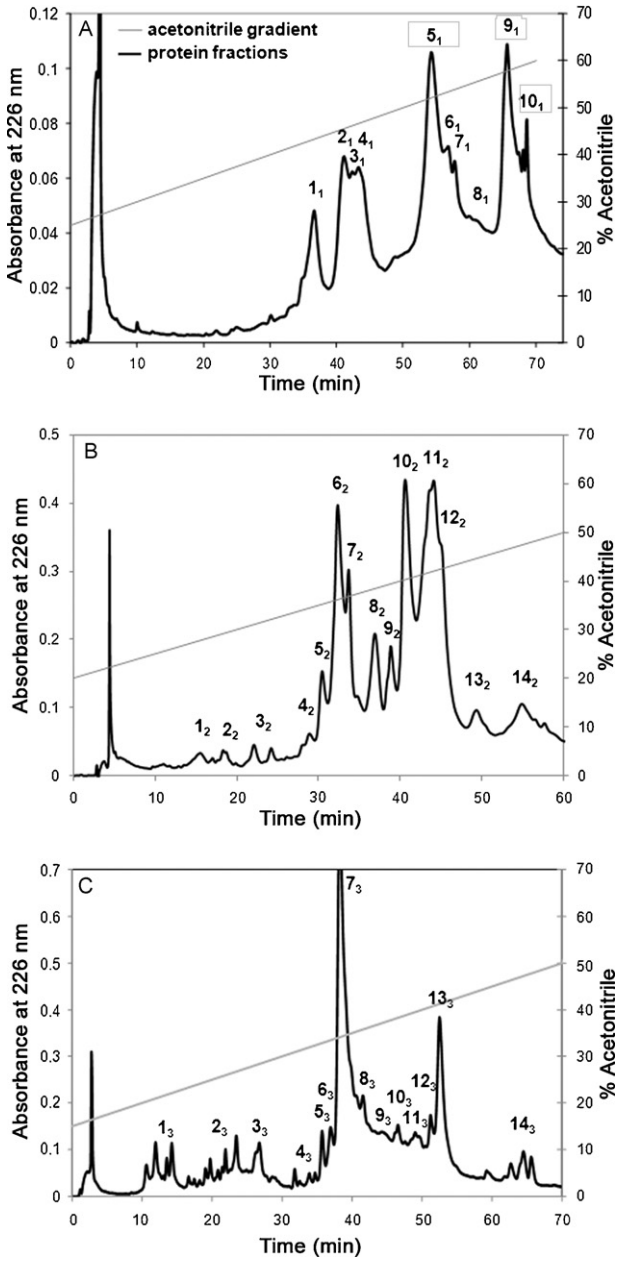

Fig. 2. Fractionation of boar epididymal fluid fractions after size exclusion chromatography of EF1–3 by high-performance liquid chromatography with reverse phase column (RP HPLC): (A) EF1 fraction, (B) EF2 fraction, (C) EF3 fraction.

exclusion chromatography (Fig. 1B). In HPLC-separated fractions of EF1, protein bands with molecular mass between 12 and 30 kDa were additionally observed and fraction EF2 displayed proteins smaller than 10 kDa.

3.3. Changes in interaction of spermatozoa and biotin-labeled ligands after incubation with epididymal fluid fractions

Binding studies on microtiter plates were used for testing the changes in interactions of epididymal spermatozoa with biotin-labeled ligands after pre-incubation with proteins of epididymal fluid fractions. Fresh epididymal spermatozoa were capable to bind the biotin-labeled ligands, such as heparin or hyaluronic acid, and glycoproteins of zona pellucida (gpZP), substances present in the natural environment of spermatozoa and important for successful realization of reproductive process. After the binding of epididymal fluid fractions EF1–3 to the sperm surface, the intensity of interactions with the biotin-labeled ligands was mostly changed (Fig. 4). No change was observed in the case of proteins from fraction EF1 and in the binding of biotin-labeled heparin or hyaluronic acid to the sperm. Increased interaction of ligands to spermatozoa was shown after the pre-incubation of sperm with the EF2 and EF3 epididymal fluid fractions. On the contrary, after the sperm incubation with proteins from fraction EF1 the binding of biotin-labeled glycoproteins of ZP to spermatozoa was decreased.

3.4. Characterization of heparin-binding fractions from epididymal fluid

Epididymal fluid fractions EF1–3 were subjected to the heparin-Sepharose affinity column. Portions of proteins non-interacted and interacted with heparin (Hep^-:Hep^+) were found in fractions EF1–3 in following amounts: 1:1 (EF1), 2:1 (EF2) and 6:1 (EF3). The largest portion of heparin-binding proteins was isolated from the EF1 epididymal fluid fraction. Heparin- and non-heparin-binding proteins were characterized by SDS-PAGE (Fig. 5). In the heparin-adsorbed fraction of EF1 (1^+), protein of Mr approx. 75 kDa was found as dominant heparin-binding protein. In fraction 2^+, major disclosed heparin-binding proteins were proteins with molecular masses around 28, 75 and 16 kDa. Heparin-binding proteins in fraction 3^+ were shown in protein bands of 16, 65 and 75 kDa.

3.5. Interaction of epididymal proteins with biotin-labeled ligands

HPLC-separated protein fractions after SDS-electrophoresis transferred to nitrocellulose membranes were tested with biotin-labeled ligands: heparin (Fig. 6A), hyaluronic acid (Fig. 6B) and glycoproteins of zona pellucida (gpZP) (Fig. 6C). Only one protein from the EF1 fraction with Mr 28 kDa showed interaction with heparin and hyaluronic acid. The affinity of some low-molecular-mass epididymal proteins (12–17 kDa and 23 kDa) to heparin and hyaluronic acid suggests their binding to oviductal proteoglycans at the sperm capacitation. Proteins in EF2 and EF3 fractions with molecular masses of 12–18 kDa interacted with ZP glycoproteins (Fig. 6C). Proteins with the ability to interact with labeled ligands characterized by relative molecular masses are summarized in Table 1. Some of these proteins were determined by N-terminal amino acid sequencing and identified.

3.6. Identification of proteins with binding activity

Table 1 represents the molecular-mass characterization and N-terminal amino acid sequencing determination of proteins present in HPLC-separated fractions of epididymal fluid EF1–3 fractions interacting with biotin-labeled ligands. As heparin-binding proteins, we detected epididymal secretory protein HE1 and epididymal secretory glutathione peroxidase (GPX5). Protein HE1 was found in two protein bands of fraction 8_2 with molecular masses 13 kDa and 17 kDa. Glutathione peroxidase was identified in fraction 11_2 with Mr 14 kDa. Protein bands of Mr 23 kDa in fractions 10_2 and 8_3 were determined as Crisp1 and HE12 proteins, respectively. Crisp1 (cysteine-rich secretory protein 1) and HE12 (epididymal sperm-binding protein) were able to bind hyaluronic acid. Lactoferrin fragment with Mr 28 kDa was interacted with both the biotin-labeled ligands, heparin and hyaluronic acid. As zona pellucida glycoprotein-binding protein, Crisp3-like protein (protein similar to cystein-rich secretory protein) was identified in the band of Mr 16 kDa in fraction 6_2.

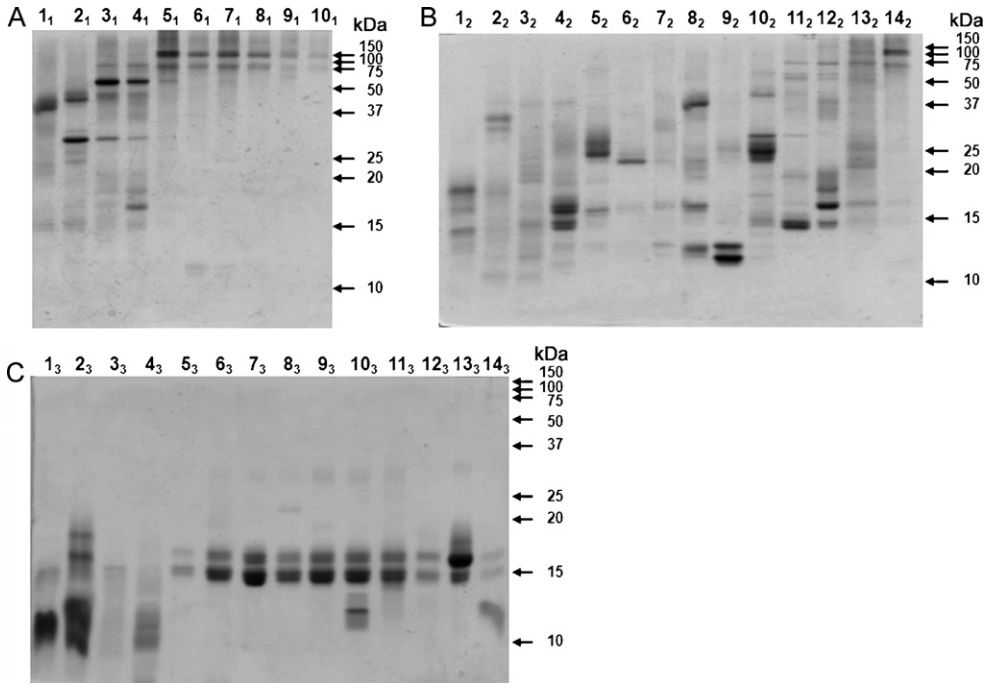

Fig. 3. 15% SDS-PAGE characterization of HPLC-separated epididymal fluid fractions EF1–3: (A) EF1 fraction, (B) EF2 fraction, (C) EF3 fraction.

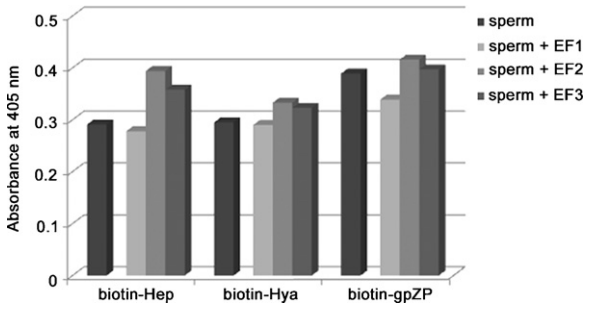

Fig. 4. Enzyme-linked binding assay (ELBA): study of changes in the interaction of boar epididymal spermatozoa with biotin-labeled ligands after the sperm incubation with epididymal fluid fractions EF1–3. Concentration of biotin-labeled ligands was 12 μg/ml in PBS; interaction was measured as absorbance of colored substrate product of enzymatic reaction at 405 nm. biotin-Hep – biotin-labeled heparin; biotin-Hya – biotin-labeled hyaluronic acid; biotin-gpZP – biotin-labeled glycoproteins of zona pellucida.

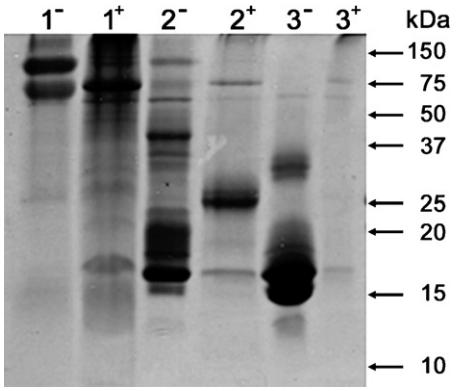

Fig. 5. 15% SDS-PAGE characterization of heparin-binding and non-heparin-binding proteins of boar epididymal fluid fractions EF1–3 obtained by affinity chromatography on immobilized heparin Sepharose column: 1^-, 2^-, 3^- – non-heparin-binding proteins of EF1–3; 1^+, 2^+, 3^+ – heparin-binding proteins of EF1–3.

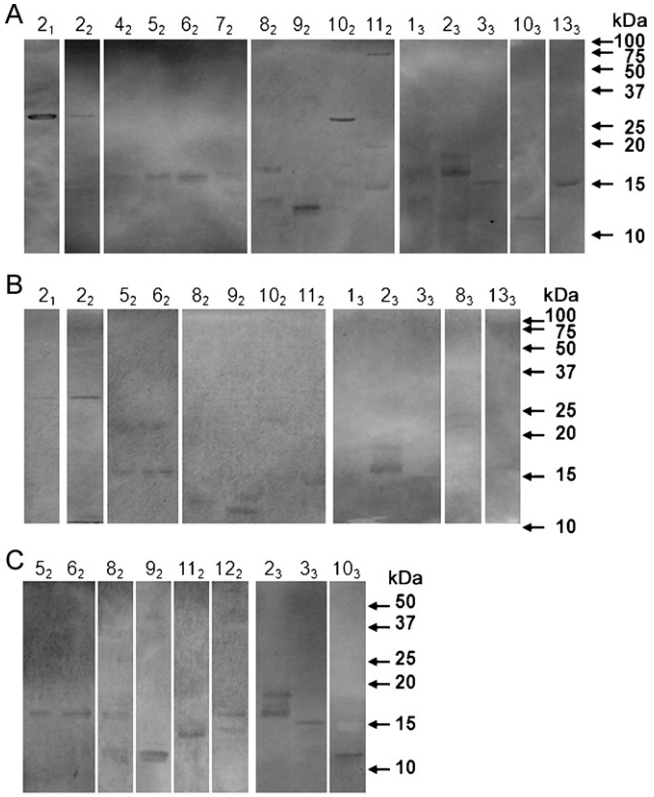

Fig. 6. Far Western blot: interaction of boar epididymal fluid proteins separated by size exclusion chromatography (EF1–3) followed by RP HPLC with biotin-labeled ligands: (A) heparin, (B) hyaluronic acid and (C) glycoproteins of zona pellucida.

4. Discussion

The epididymis is an important reproductive organ where spermatozoa gain the fertilizing and movement abilities. During the maturation in the epididymis, spermatozoa are directly exposed to epididymal fluid. Components of the fluid have an immediate effect on the sperm maturation or they might bind to the sperm surface and play a role in subsequent steps of reproduction. The aim of our study was to separate and characterize proteins of boar epididymal fluid and to monitor their protein binding abilities. The fluid was isolated from the end of epididymal tube and proteins were separated into three fractions (EF1–3) according to their molecular masses. Epididymal fractions EF1–3 were further fractionated by HPLC with reverse phase and then characterized by SDS-PAGE. By the ELBA method and Far Western blot analysis with biotin-labeled ligands, we investigated the binding ability of EF1–3 protein fractions and HPLC-separated proteins of epididymal fluid. We studied the changes in the binding ability of epididymal spermatozoa to biotin-labeled heparin, hyaluronic acid, and glycoproteins of zona pellucida (gpZP). Heparin and hyaluronan are present in the lumen of mammalian oviduct as a part of glycosaminoglycans and contribute to the sperm capacitation [27,28]. Proteins attached to the sperm surface during the transit through the epididymis could affect the sperm-binding ability and play a role in successful realization of the reproductive process. Epididymal proteins represent the first coating layer on the sperm surface before seminal plasma attachment [8]. Many proteins leave the sperm surface during capacitation and receptors for ZP binding are exposed [1]. In ELBA experiments, we observed that the ZP glycoproteins bound the sperm with the highest affinity. We noticed changes in the interaction of epididymal spermatozoa with biotin-labeled ligands after pre-incubation with proteins of epididymal fluid fractions. After the binding of epididymal fluid fractions EF1–3 to the sperm surface, the intensity of interactions with the biotin-labeled ligands was mostly changed (Fig. 4). No changes were observed with proteins from the EF1 fraction in the binding of biotin-labeled heparin and hyaluronic acid to the sperm. On the contrary, the interaction of biotin-labeled glycoproteins of ZP to spermatozoa was decreased. Increased interaction of ligands to spermatozoa was shown after pre-incubation of sperm mainly with the EF2 epididymal fluid fraction. Based on this experiment we conclude that the EF2 fraction contains most of the proteins with binding ability to the tested ligands. On the other hand, the EF1 fraction includes most proteins that predominantly cover the ligand-binding sites on the sperm surface.

For more detailed characterization of heparin-binding proteins in epididymal fluid, we carried out the affinity chromatography on immobilized heparin under physiological conditions. By this method, the largest portion of heparin-binding proteins was obtained in the EF1 fraction. This result does not correspond with the ELBA experiment, which showed the highest increase of heparin-binding sites on sperm after pre-incubation with proteins from the EF2 fraction (Fig. 4). In case of the ELBA assay, not many proteins with heparin-binding activity from fraction EF1 were attached to the sperm surface. Heparin-adsorbed proteins on the affinity column were characterized by SDS-PAGE. As heparin-binding proteins, protein with molecular mass of 75 kDa was found in fraction 1^+ and proteins of 28 and 16 kDa in fractions 2^+ and 3^+. The high-molecular-mass protein (75 kDa) was not obtained as heparin-binding protein in the following experiment with HPLC-separated proteins. This indicates that the interaction of proteins with heparin during the affinity-column experiment might not occur directly, but via other proteins. Primarily, proteins binding to heparin were adsorbed on the affinity column and then proteins which interacted with these proteins were sequentially captured.

Table 1
Molecular-weight characterization and sequence determination of HPLC-separated epididymal fluid proteins interacted with biotin-labeled ligands.

	Biotin-labeled heparin	Biotin-labeled hyaluronic acid	Biotin-labeled gpZP
EF1	28 kDa (2_1) (LF)	28 kDa (2_1) (LF)	–
EF2	12 kDa (9_2)	12 kDa (9_2)	12 kDa (8_2, 9_2)
	13 kDa (8_2) (HE1)	13 kDa (8_2)	14 kDa (11_2)
	14 kDa (11_2) (GPX5)	14 kDa (11_2)	15 kDa (12_2)
	16 kDa (2_2, 4_2, 5_2, 6_2, 7_2)	16 kDa (5_2, 6_2)	16 kDa (5_2, 6_2, 8_2) (Crisp3-like)
	17 kDa (8_2) (HE1)	23 kDa (5_2, 6_2, 10_2) (Crisp1)	17 kDa (12_2)
	19 kDa, 75 kDa (11_2)	28 kDa (2_2)	
	28 kDa (2_2, 10_2)		
EF3	11 kDa (2_3, 10_3)	15 kDa (1_3, 3_3)	12 kDa (10_3)
	13 kDa (1_3)	16 kDa (2_3, 13_3)	15 kDa (3_3)
	15 kDa (3_3, 13_3)	18 kDa (2_3)	16 kDa (2_3)
	16 kDa (1_3, 2_3)	23 kDa (8_3) (HE12)	18 kDa (2_3)
	18 kDa (2_3)	25 kDa (8_3)	

Proteins from individual HPLC-separated fractions determined by N-terminal amino acid sequencing and identified proteins: LF – lactoferrin fragment (APKKG...), HE1 (NPC2) – epididymal secretory protein 1 (EPVHFR...), GPX5 – epididymal secretory glutathione peroxidase (ASNLE...), Crisp1 – cysteine-rich secretory protein 1 (KPAQVPYKTLLT...); Crisp3-like – similar to cysteine-rich secretory protein 3 (YAVGPXKPPTXE...); HE12 – epididymal sperm-binding protein (human epididymal gene hE12) (DTKDSXVFPAFY...).

Further chromatographic separation (RP HPLC) of epididymal fluid and binding assay on blots with biotin-labeled ligands brought additional information about the binding ability of epididymal fluid proteins. Some of epididymal proteins with ligand-binding ability were identified (Table 1). As heparin and hyaluronan binding proteins, HE1 and HE12 were identified in fractions 8_2 and 8_3 (Table 1), respectively. The protein expression of HE1 was found along the complete boar epididymis; however, the expression of HE12 only in the corpus and cauda parts. Moreover, both proteins HE1 and HE12 have been immunodetected in the extract from epididymal spermatozoa [14]. We identified the HE1 protein in boar epididymal fluid in fraction 8_2 as bands of Mr 13 and 17 kDa characterized by N-terminal amino acid sequencing (Table 1). Porcine HE1 has been described as secreted glycoprotein; its saccharide chain is processed from the form of 19–16 kDa during transit through the epididymis [29]. We found forms with lower Mr; these proteins have probably been truncated at the C-terminus. HE1 was found to possess cholesterol-binding activity and is considered to be one of the important regulators of cholesterol content in the sperm membrane. However, its role in sperm maturation and capacitation in connection with cholesterol balance in the sperm membrane is not clear [29]. Our results showing the HE1 binding ability to heparin might suggest a role for this protein in the sperm capacitation. Latest study has shown the capacitating effect of heparin *in vitro*. An increase in the number of boar capacitated sperm, probably due to an accelerating effect on the sperm metabolism, has been observed after the sperm incubation with heparin added into the capacitation medium [30]. Human HE12 protein has been characterized as a protein with four fibronectin type II-modules (Fn2) [31]. The same domains have been described previously in bovine seminal plasma proteins [32]. Bovine seminal plasma protein PDC-109 is able to extract cholesterol from the sperm plasma membrane, process linked to the Fn2 protein-mediated initiation of the sperm capacitation in cattle [33]. The localization of porcine HE12 on the sperm was determined in the acrosomal region [34] and its role in capacitation in the pig may be supposed. We found HE12 protein (23 kDa) in epididymal fluid

(fraction 8_3) with the ability to bind hyaluronic acid. Other known epididymal proteins belong to the cysteine-rich secretory protein (Crisp) family [35]. In pig, the *Crisp* genes have been described by Vadnais et al. [36]. The *Crisp1* gene is expressed by the epididymal tissue and *Crisp3* mainly by prostate, but a weak signal was also noticed in the testis and caput epididymis [36]. In boar epididymal fluid, N-terminal amino acid sequence analysis showed the protein fragment (Mr 16 kDa) interacting with biotin-labeled zona pellucida glycoproteins as similar to the Crisp3 protein. Crisp3 has been described in horse to be attached to the sperm surface and to be positively correlated with fertility [37]. Glutathione peroxidase (GPX) and lactoferrin have been found on the sperm in various species. The function of these proteins is potentially the same for the soluble and bound forms and they might provide a protective effect against peroxidation of membranes [9]. Based on our results, the ability of lactoferrin fragment and GPX to interact with heparin and hyaluronan suggests their role as sperm decapacitation factors. Hyaluronan and heparin proved as part of glycosaminoglycans in porcine oviductal fluid seem to be involved in sperm survival, capacitation and binding to and release from the sperm reservoir [30,38].

Altogether, our experiments showed that some epididymal proteins likely bind the spermatozoa and affect the binding sites on the sperm surface. We determined and identified some proteins from boar epididymal fluid with affinity to heparin, hyaluronan and ZP glycoproteins. These findings indicate that epididymal fluid proteins bind to the sperm surface during epididymal maturation and might subsequently play roles in the sperm capacitation or sperm–zona pellucida binding. However, further studies should explain which particular epididymal proteins with ligand-binding abilities attach to the sperm during epididymal transport and participate in fertilization.

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