Spermadhesins of the AQN and AWN families, DQH sperm surface protein and HNK protein in the heparin-binding fraction of boar seminal plasma

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Heparin-binding proteins (designated BHB-2–BHB-9) were isolated from boar seminal plasma by affinity chromatography on heparin immobilized on polyacrylamide gel, followed by reverse phase HPLC. According to their N-terminal amino acid sequences, BHB-3–BHB-5 belong to the AQN family of spermadhesins and BHB-7–BHB-9 to the AWN family. BHB-6 is composed of two different proteins. The dominant protein (14 kDa) has the N-terminal amino acid sequence HNKQEGRDHD that is identical to the sequence of human semenogelin at positions 85–94. The minor proteins (16 and 17 kDa) belong to the AWN family of spermadhesins. The 14 kDa HNK protein does not crossreact with antibodies against AQN or AWN spermadhesins. BHB-2 also binds to the acrosome of boar epididymal spermatozoa but has the N-terminal sequence DQH. Therefore, basic protein BHB-2 belongs to a new family of DQH sperm surface proteins that are homologous to the acidic proteins from bull and stallion seminal plasma, to the collagen binding domain II in fibronectin and to the leucocyte cell–cell adhesion regulator, but are not homologous to AQN or AWN spermadhesins. Nevertheless, anti-AQN-1 spermadhesin antibodies crossreact strongly with DQH protein. All boar heparin-binding proteins bind concanavalin A indicating their glycoprotein nature, which was proved by the detection of glucosamine and galactosamine residues in their molecules. Furthermore, spermadhesins interact with zona pellucida, protease inhibitors and a polyacrylamide derivative of heparin. Affinity chromatography experiments showed that the DQH protein bound to gelatin–agarose together with the AWN proteins and that the DQH protein and AQN-1 spermadhesin belong to the phosphoryl choline binding proteins.

Introduction

Mammalian fertilization is a complex process involving a series of events that include sperm capacitation, gamete recognition and binding to the ovum. A series of interactions between molecules located on the surface of both gametes participate in the individual events of fertilization.

Boar spermadhesins are multifunctional proteins located on the sperm surface (Jonáková *et al.*, 1995; Töpfer-Petersen *et al.*, 1995) that participate in various types of interaction either with components of the zona pellucida or with substances that are present in the natural environment of both gametes. Some physicochemical and binding properties of spermadhesins of the AQN and AWN families and porcine seminal plasma protein I (PSP-I) spermadhesin have already been described (Calvete *et al.*, 1993a; Kwok *et al.*, 1993; Sanz *et*

al., 1993; Jonáková *et al.*, 1995; Liberda *et al.*, 1997a; Tichá *et al.*, 1998). Some spermadhesins interact with the zona pellucida (Jonáková *et al.*, 1991; Parry *et al.*, 1992; Calvete *et al.*, 1993a; Töpfer-Petersen *et al.*, 1995). In addition, some have an affinity for heparin and bind protease inhibitors (Parry *et al.*, 1992; Sanz *et al.*, 1992; Calvete *et al.*, 1993b). Previous studies showed that the binding properties of AQN and AWN spermadhesins depend on their glycosylation (Calvete *et al.*, 1993a; Calvete *et al.*, 1993b). The complete amino acid sequences of several spermadhesins have been reported (AQN-1, AQN-3, AWN, PSP-I, PSP-II, bovine acid seminal fluid protein (aSFP)). These proteins are composed of 109–133 amino acid residues, contain two conserved disulphide bridges and have 40–60% amino acid sequence identity (reviewed in Töpfer-Petersen *et al.*, 1995).

The present study deals with a detailed characterization of separated proteins of the heparin-binding fraction of boar seminal plasma. Attention was focused on comparison of the immunological and binding properties of spermadhesins of

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the AQN and AWN subfamilies with those of DQH sperm surface protein and HNK protein to gain a better understanding of the role of these spermadhesins *in vivo*.

Materials and Methods

Materials

Boar semen was obtained from healthy Large White pigs from the Insemination Station, Nové Mlýny. Pig ovaries (Large White), pig blood serum, boar cauda epididymal fluid and mice spleens were obtained from slaughtered animals and stored frozen at -70°C . Boar seminal plasma was isolated from sperm ejaculate by centrifugation at 600 g for 20 min (5°C). Boar cauda epididymal spermatozoa were obtained from the epididymal fluid of slaughtered animals. Spermatozoa were washed three times with PBS ($\text{pH } 7.2$, $0.15 \text{ mol phosphate l}^{-1}$, $0.3 \text{ mol NaCl l}^{-1}$) to remove epididymal fluid, followed by centrifugation at 600 g for 5 min (5°C).

Heparin and bovine trypsin kallikrein inhibitor (TKI) were obtained from Léčiva (Praha). Avidin, avidin-peroxidase, fluorescein isothiocyanate (FITC), 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), *N*-hydroxysuccinimidobiotin, gelatin-agarose and phosphoryl choline were purchased from Sigma (St Louis, MO). Soya bean Kunitz trypsin inhibitor (STI) was purchased from Boehringer Mannheim (Mannheim). Concanavalin A was obtained from the Laboratory for Production and Control of Lectin Preparations (Praha). 4-Chloro-1-naphthol was supplied by Serva (Heidelberg) and *o*-phenylenediamine was obtained from Fluka (Buchs).

Nitrocellulose membranes (RPN 203G Hybond C-Super) were obtained from Amersham (Amersham). Dialysis membranes (Spectra/PorR Membrane MWCO 6-8000) were supplied by Roth (Karlsruhe). Servalyt Pre Cotes and Servalyt Pre Nets, both $\text{pH } 3-10$, the pI calibration kit ($\text{pH range } 3-10$) and the low molecular mass protein standards were purchased from Serva (Heidelberg).

Immobilization of heparin was achieved by coupling the polysaccharide to poly(acrylamide-allylamine) copolymer using the carbodiimide reaction as described by Tichá *et al.* (1994). A water soluble poly(acrylamide-allylamine) derivative of heparin was prepared using the method of Klein *et al.* (1995). The reaction described by Liberda *et al.* (1997b) was used for the preparation of a fluorescein-labelled derivative of avidin (FITC-avidin).

Purification of heparin-binding proteins

Boar seminal plasma stored at -20°C was thawed and centrifuged for 10 min at 10 000 g. The seminal plasma was then diluted 1:1 with PBS ($\text{pH } 7.3$, $20 \text{ mmol phosphate l}^{-1}$ containing $150 \text{ mmol NaCl l}^{-1}$) and applied (50 ml) to a heparin-polyacrylamide column ($30 \text{ mm} \times 75 \text{ mm}$) pre-equilibrated with the same buffer (Tichá *et al.*, 1994). Non-adsorbed proteins were washed off with the starting buffer and the bound proteins were eluted with $3 \text{ mol NaCl l}^{-1}$.

Fractions containing heparin-binding proteins (designated BHB-2–BHB-9) were pooled, desalted on Sephadex G-25 (Pharmacia, Uppsala) in 0.2% acetic acid and lyophilized. Further purification of heparin-binding proteins was achieved by reverse phase HPLC on a semipreparative 218 TP 1010 Vydac C_{18} column ($10 \text{ mm} \times 250 \text{ mm}$, $10 \mu\text{m}$ particle size). 5 mg of protein preparation in 1 ml 0.05% trifluoroacetic acid was applied to the column and the separated proteins were eluted with linear gradients of 30–60% acetonitrile (75 min) followed by 60–80% acetonitrile (15 min). Rechromatography of BHB-2, BHB-3, BHB-6 and BHB-7 was carried out on an analytical 218 TP 104 Vydac C_{18} column ($4.6 \text{ mm} \times 250 \text{ mm}$, $5 \mu\text{m}$ particle size) in 0.05% trifluoroacetic acid in water, with a linear gradient of either 20–40% acetonitrile (60 min, BHB-2 and BHB-3) or 25–40% acetonitrile (50 min, BHB-6 and BHB-7). Protein fractions were lyophilized.

Affinity chromatography

Gelatin-agarose. Heparin-binding fraction (10 mg) was applied to a gelatin-agarose column ($1 \text{ cm} \times 5 \text{ cm}$) equilibrated with PBS ($\text{pH } 7.4$). Non-adsorbed proteins were eluted with the starting buffer; adsorbed proteins were eluted with $4 \text{ mol urea l}^{-1}$ in PBS at a flow rate of $3 \text{ ml (20 min)}^{-1}$. Fractions containing proteins (as determined by absorbance at 280 nm) were collected, desalted on Sephadex G-25 in 0.2% acetic acid and lyophilized. The protein preparations obtained were analysed by reverse phase HPLC and SDS-PAGE.

Heparin-polyacrylamide gel followed by phosphoryl choline elution. Boar seminal plasma (50 ml) was applied to the heparin-polyacrylamide gel column ($1.5 \text{ cm} \times 15 \text{ cm}$) equilibrated with $0.05 \text{ mol Tris-HCl l}^{-1}$ buffer ($\text{pH } 7.4$, containing $0.15 \text{ mol NaCl l}^{-1}$) as described by Tichá *et al.* (1994). The non-adsorbed proteins were eluted with the same buffer; the adsorbed proteins were first eluted with $0.05 \text{ mol Tris-HCl l}^{-1}$ buffer (containing $0.15 \text{ mol NaCl l}^{-1}$ and $0.01 \text{ mol phosphoryl choline l}^{-1}$, $\text{pH } 7.4$) and then with $3 \text{ mol NaCl l}^{-1}$ at a flow rate of $3 \text{ ml (15 min)}^{-1}$. Further preparation and analysis of the protein fractions were carried out as described for the affinity chromatography on gelatin-agarose.

Preparation of solubilized zona pellucida

Oocytes were released from pig ovaries (either fresh or frozen) in a commercial meat grinder with a large amount of ice-cold saline ($0.15 \text{ mol NaCl l}^{-1}$) and the homogenate was sieved through nylon screens as described by Hedrick and Wardrip (1986). The oocytes were separated from other particles by centrifugation in a discontinuous Percoll gradient according to the method of Hokke *et al.* (1994). The oocytes were collected from the 10–20% interface, washed with distilled water and gently homogenized using a small glass homogenizer. The zona pellucida 'ghosts' were then collected on a $40 \mu\text{m}$ screen and repeatedly washed with saline. Isolated zonae pellucidae were heat solubilized in

0.2 mol NaHCO_3 l^{-1} (pH 9.0) at 73°C for 30 min and centrifuged at 350 g for 10 min (4°C). The clear supernatant with a protein concentration of 0.32 mg ml^{-1} was used for biotinylation.

Preparation of antisera

Rabbit antiserum to both AQN-1 and AWN-1 spermadhesins was prepared by immunizing female rabbits with an intracutaneous injection of 1 mg highly purified protein dissolved in 0.1 ml of 0.15 mol NaCl l^{-1} mixed with Freund's complete adjuvant. At intervals of 2 and 4 months after the initial immunization, the rabbits were injected with 500 μg of the antigen in Freund's complete adjuvant. Two weeks after the last injection, the animals were bled through the ear vein. The antisera were heat inactivated for 30 min at 56°C . After immunoelectrophoresis (Williams, 1971), a single precipitation line was seen in the reaction of antiserum to AQN-1 and AWN-1 with the corresponding antigen. The specificity of both antisera was determined by ELISA. In both cases, preincubation (2 h at 37°C and overnight at 4°C) of 200 μg of the antigen (AQN-1 or AWN-1) with 0.1 ml of the corresponding antiserum (anti-AQN-1 or anti-AWN-1, respectively) resulted in a negative reaction.

Preparation of splenocytes

Mice spleens were homogenized in PBS (pH 7.2) and the cell suspension was passed through a 110 μm mesh. The suspension was then washed by centrifugation in PBS at 400 g (3×5 min) and adjusted to 5×10^6 cells ml^{-1} PBS containing 250, 100, 20 or 1 μg of BHB-2. After 30 min incubation at 37°C , a drop of the cell suspension was placed onto a microscope slide and cell agglutination was evaluated under the microscope.

Amino acid analysis and N-terminal sequence determination

Amino acid analyses were carried out using a Biochrom 20 analyser (Pharmacia Biotech, Uppsala) after sample hydrolysis with 6 mol HCl l^{-1} at 110°C for 20 h. For quantitation of free cysteine residues and disulphide bridges in BHB-2, the protein was treated with iodoacetamide either directly or after reduction (boiled in 1% 2-mercaptoethanol) (see Einspanier *et al.*, 1994). Carboxymethylcysteine was determined after hydrolysis for 24 h. For amino sugar analyses, the samples were hydrolysed with 4 mol HCl l^{-1} at 110°C for 4 h and analysed using a T339 amino acid analyser (Mikrotechna, Praha). N-terminal amino acid sequences were determined automatically on the Applied Biosystems 470 A protein sequencer. Program 02R PTH was used and phenylthiohydantoin derivatives of the amino acid residues were analysed by HPLC (Tsunasawa *et al.*, 1985). N-terminal acid sequencing of BHB-2 and the proteins from BHB-6 was performed using a gas-liquid phase protein sequencer (Beckman LF 3600, Fullerton, CA) following the manufacturer's instructions. Searches for amino acid

similarities were carried out using the protein sequence deposits in the BLAST-BASIC e-mail server databank (Altschul *et al.*, 1990).

Electrophoresis and blotting

SDS-PAGE was carried out on 15% slab gels as described by Laemmli (1970) and blotting onto nitrocellulose sheets was carried out as described by Towbin *et al.* (1979). The molecular mass of separated proteins was estimated by comparison with low molecular mass protein standards run in parallel.

Solid phase protein-binding assay on nitrocellulose sheets was carried out using the following procedure. After blotting, the proteins separated by electrophoresis were deactivated in buffer (0.01 mol Tris-HCl l^{-1} (pH 7.4), 0.85% (w/v) NaCl , 3% (w/v) BSA, 0.02% (v/v) Tween 20) for 2 h at 22°C . The blots were then incubated for 2 h at 37°C with biotinylated zona pellucida (5 μg ml^{-1}) in washing buffer (0.01 mol Tris-HCl l^{-1} (pH 7.4), 0.85% (w/v) NaCl , 3 mmol CaCl_2 l^{-1}), washed, and incubated for 1 h at 37°C with avidin-peroxidase in buffer (0.1 mol Tris-HCl l^{-1} (pH 8.0) 1% (w/v) BSA). The blots were then washed again and developed at 20°C in the dark by treatment with 0.05% 4-chloro-1-naphthol in 0.01 mol Tris-HCl l^{-1} (pH 7.4) containing 0.001% (w/v) CoCl_2 and 0.09% (v/v) H_2O_2 . The reaction was stopped after 5 min by washing the blots in distilled water.

Isoelectric focusing

Isoelectric focusing (IEF) was carried out on a FBA 3000 (Pharmacia, Uppsala) using polyacrylamide layer Servalyt Pre Cotes 3–10 and a pI calibration kit (pH 3–10). The conditions for IEF were as described in the manufacturer's manual. Proteins were stained with Coomassie brilliant blue R-250. Servalyt Pre Nets 3–10 were used for electroblotting of separated proteins to nitrocellulose. Proteins resolved by IEF were transferred immediately to a nitrocellulose membrane in a semi-dry electroblotter (Omni-Trans, Omni-Bio, Brno). Electrotransfer was conducted at a current density of 0.8 A cm^{-2} in 0.05 mol citrate phosphate l^{-1} (pH 4.6) or in 0.05 mol Tris-HCl l^{-1} (pH 8.5) for 90 min.

Biotinylation of sperm proteins and ligands

The biotinylation of a water-soluble poly(acrylamide-allylamine) derivative of heparin was as described by Klein *et al.* (1995). The spermadhesins BHB-2, BHB-3 and BHB-7, solubilized zona pellucida, bovine TKI and STI were incubated with 0.4% *N*-hydroxysuccinimidobiotin in dimethylformamide for 30 min at 25°C . The reaction was stopped by addition of 1 mol NH_4Cl l^{-1} to adjust the pH to 6.0. The biotinylated proteins were dialysed on Spectra/Por MWCO 6/8000 membranes against 1 mmol HCl l^{-1} and were used immediately. Biotinylated zona pellucida (stock solution 220 μg ml^{-1}) was dialysed against 20 mmol Tris-HCl l^{-1} (pH 7.8, 0.4 mol NaCl l^{-1}) and stored at -20°C .

Enzyme-linked binding assay (ELBA)

For the ligand-binding assay, the proteins (1.0–2.5 µg) were immobilized on microtitration plates (Dynatech, Chantilly, VA) in 100 µl of coating buffer (0.05 mol NaHCO₃ l⁻¹, pH 9.6) by incubation overnight at 4°C. After rinsing with washing buffer (0.01 mol Tris HCl l⁻¹ (pH 7.4) 0.85% (w/v) NaCl, 3 mmol CaCl₂ l⁻¹, 0.5% (w/v) BSA), the wells were deactivated with 250 µl of 3% (w/v) BSA in washing buffer (2 h at 4°C). After washing, biotinylated water soluble polyacrylamide derivative of heparin (5 µg), biotinylated solubilized zona pellucida (0.5 µg), or proteinase inhibitor (2 µg), each in 100 µl of washing buffer, was applied to the assay wells and incubated for 2 h at 37°C. After washing, 25 ng avidin–peroxidase in 100 µl of 0.1 mol Tris–HCl l⁻¹ (pH 8.0) containing 1% (w/v) BSA was added and incubated for 1 h at 37°C. After washing, the enzyme reaction was initiated by the addition of 250 µl 0.01% (w/v) ABTS in 0.1 mol citrate phosphate l⁻¹ (pH 4.6) in the presence of 0.09% (v/v) H₂O₂ into each well and developed at 37°C. Absorbance was measured at 405 nm after 30 min using a microplate reader (SLT-Spectra, SLT-Labinstruments, Vienna).

Enzyme-linked immunosorbent assay

Wells in the assay plates were primed overnight at 4°C with antigens (1.0–2.5 µg (100 µl)⁻¹) in coating buffer (0.05 mol NaHCO₃ l⁻¹, pH 9.6). After rinsing with washing buffer (PBS (pH 7.2) containing 0.5% (w/v) BSA and 0.05% (v/v) Tween 20 or 2% (v/v) calf serum), the wells were deactivated with 250 µl of 3% (w/v) BSA in washing buffer for 2 h at 4°C. After further rinsing with washing buffer, the antisera to spermadhesins (serial dilutions in PBS–Tween) were added to each well, and the plates were incubated at 22°C for 1 h. After thorough washing, the spermadhesin antibodies were reacted with peroxidase-labelled goat anti-rabbit globulin in washing buffer (1:5000; 100 µl per well) at 22°C for 30 min, washed again, and the bound peroxidase activity was detected using 0.5% (w/v) *o*-phenylenediamine and 0.03% (v/v) H₂O₂ as a substrate in 0.1 mol citrate phosphate l⁻¹ (pH 5.5). The reaction was stopped after 30 min with 4.5 mol H₂SO₄ l⁻¹ (50 µl per well), and the absorbance was determined at 492 nm using an ELISA reader.

Sperm-binding activity

The sperm-binding activity of biotinylated BHB-2 protein was tested with epididymal spermatozoa. The washed epididymal spermatozoa (1 × 10⁵ cells per well) were immobilized on plates in 100 µl of coating buffer (0.05 mol NaHCO₃ l⁻¹, pH 9.6) overnight at 4°C. The immobilized spermatozoa were incubated with serially diluted biotinylated BHB-2 protein (2.5 µg (100 µl)⁻¹) dissolved in washing buffer (100 µl per well) for 2 h at 37°C. The spermatozoa were then washed and treated with avidin–peroxidase in washing buffer (25 ng (100 µl)⁻¹) for 1 h at 37°C. After further washing, the enzyme reaction was developed for 30 min at 37°C with ABTS using the

procedure described above for the ELBA. The absorbance was measured at 405 nm.

Binding of boar heparin-binding proteins to epididymal spermatozoa was studied using a fluorescence microscope (Leitz Orthoplan, Vienna). Epididymal spermatozoa were smeared on clean glass slides, air-dried and then preincubated for 30 min at 37°C in PBS containing 100 or 50 µg heparin-binding protein ml⁻¹. After washing (PBS, 0.2% (w/v) BSA), spermatozoa were incubated for 1 h at 22°C with FITC–avidin (70 µg PBS ml⁻¹). The epididymal spermatozoa used as controls were prepared in the same way but without heparin-binding protein.

Results

Isolation and characterization

Heparin-binding proteins from boar seminal plasma were isolated by affinity chromatography on heparin immobilized on polyacrylamide gel. Individual proteins were separated by reverse phase HPLC and eight fractions (designated BHB-2–BHB-9) were obtained and characterized by relative molecular mass (Fig. 1). Rechromatography was used for further purification of BHB-2, BHB-3, BHB-6 and BHB-7. Purified protein fractions were characterized by amino acid analysis and N-terminal sequence determination (Table 1). Most of the proteins are of basic character with relatively high isoelectric points (pI values in the range 8.5–9.3), with the exception of the BHB-6 fraction (Table 1). Single N-terminal amino acid sequences were determined for all of the protein fractions obtained, with the exception of BHB-6 and BHB-9. These results showed that proteins in the BHB-3–BHB-5 fractions belong to the AQN family of spermadhesins, and proteins in the BHB-7–BHB-9 fractions belong to the AWN family of spermadhesins (Jonáková *et al.*, 1991; Sanz *et al.*, 1993). One protein in each of the BHB-6 and BHB-8 fractions belongs to the AWN family. The N-terminal amino group of the 70 kDa protein of the BHB-8 fraction was blocked and was not characterized further. Similarly, the N-terminal amino group of the BHB-9 protein was blocked, but according to amino acid analysis, the protein belongs to the AWN family of spermadhesins. The second protein component of BHB-6 (*M*_r 14 kDa) possesses the N-terminal amino acid sequence HNKQEGRDHD (HNK protein) that is identical to the amino acid sequence at positions 85–94 of semenogelin (Lilja *et al.*, 1989) (Table 1).

The N-terminal amino acid structure of BHB-2 is different from other boar spermadhesins so far characterized; it starts with the sequence DQH (Table 1). Searches for amino acid similarities showed that this DQH protein is characterized by homology to the main acidic proteins of bull seminal plasma (PDC-109 and BSP-A3), to HSP-1 and HSP-2 of stallion seminal plasma, and to the adhesion inducing protein (pAIF-1) previously isolated from boar seminal vesicle fluid (Fig. 2). The amino acid composition of BHB-2 differs from those of AWN and AQN proteins, especially in the half-cysteine content. BHB-2 contains more than seven half-cysteine residues as determined by the amino acid analysis (results not shown). The iodoacetamide treatment

Fig. 1. Purification by reverse phase HPLC of boar seminal plasma proteins bound to heparin-polyacrylamide affinity column. Isolation of boar heparin-binding proteins was achieved by reverse phase HPLC on a 218 TP 1010 Vydac C_{18} column in 0.05% trifluoroacetic acid with a linear gradient of 30–60% acetonitrile (75 min) followed by 60–80% acetonitrile (15 min). Absorbance at 226 nm (—); acetonitrile gradient (-----). The inset shows SDS-PAGE of the chromatographic fractions under non-reducing conditions. Lane 0, standard molecular mass markers in kDa (bovine serum albumin, egg albumin, carbonic anhydrase, ribonuclease, cytochrome C); lanes 2–9, purified heparin-binding proteins in fractions BHB-2–BHB-9.

Table 1. N-terminal amino acid sequences of boar heparin-binding proteins obtained by reverse phase HPLC

Heparin-binding protein	Protein	M_r (kDa)	pI	N-terminal amino acid residues
BHB-2	DQH	13	8.6	1 20 DQHLPGRFLTPAITSDDKCV 21 41 FPFIYKGNLYFDCTLHDSTYY
BHB-3	AQN-1	13	9.0	1 8 AQNKGP HK
BHB-4	AQN-2	14, 16	9.3; 9.5	1 10 AQNKGSDDXD
BHB-5	AQN-3	12	9.4	1 8 AQNKGSDD
BHB-6	HNK ^a	14	6.9	1 10 HNKQEGRDHD
	AWN-1	16, 17	9.0; 9.3	1 10 AWNRRSR SXGG
BHB-7	AWN-1	14	9.3	1 10 AWNRRSR SXGG
BHB-8	AWN-1	14	8.7	1 10 AWNRRSR SXG
	P-70 ^b	70	6.4	N-terminus blocked
BHB-9	AWN-2	16	6.4	N-terminus blocked

^aDominant N-terminal sequence (66%) found in BHB-6.

^bP-70: protein that immunoreacted with anti-AWN serum.

BHB 2: ¹ **D Q H L** - - - - - **P G R F L T P A** - - - - **I T S D**
 pAIF-1: ¹ **D Q H L** - - - - - **P G R F L X P A** - - - - **I T S D**
 BSP-A3: ¹ **D Q Q L S E D N V I L P K E K K D P A** - S G A E T K D
 PDC-109: ¹ **D Q D** - - - E G V S T E P T Q D G P A - E L P E D E E
 HSP-1: ¹⁶ **D Q Q L I M** - - - - - - T K - H S **A T V T P E** - - -

BHB 2: ¹⁷ **D K C V F P F I Y K G N L Y F D C T L H D S T Y Y** ...
 pAIF-1: ¹⁷ **D K C V F P F I Y K G N L Y F D** ...
 BSP-A3: ²⁷ **N K C V F P F I Y G N K K Y F D C T L H G S L F L** ...
 PDC-109: - ⁻²⁴ **C V F P F V Y R N R K H F D C T V H G S L F P** ...
 HSP-1: ³² **N K C V F P F N Y R G Y R Y Y D C T R T D S F Y R** ...

Fig. 2. Comparison of the N-terminal sequence of boar heparin-binding protein BHB-2 with homologous sequences of leucocyte cell-cell adhesion regulator (pAIF-1) from boar seminal plasma and proteins from bull (BSP-A3 and PDC-109) and stallion (HSP-1) seminal plasma. Identical amino acid residues are shown in bold letters.

did not show the presence of any free -SH groups in the non-reduced DQH protein, whereas reduction and alkylation resulted in the formation of eight carboxymethylcysteine residues mol⁻¹ of the protein. These results provide evidence for the presence of four disulphide bridges in the DQH molecule; AQN and AWN spermadhesins contain only two. In addition, the amino acid composition of the DQH protein was characterized by a high content of tyrosine and threonine and a low content of glycine.

Amino acid analysis was also used for the determination of the galactosamine and glucosamine content; all proteins contained glucosamine, while only DQH and AWN-1 proteins contained galactosamine.

Immunological characterization of boar heparin-binding proteins

Anti-AQN-1 serum yielded a strong reaction with the AQN spermadhesins (BHB-3–BHB-5) and a weaker reaction with the DQH protein (BHB-2). The antiserum titre in the reaction with 1 µg of AQN-1 was 1:300 000 and with the same amount of DQH it was 1:120 000. Both native and reduced proteins reacted with equal intensity. No reaction was observed in the case of AWN proteins. Anti-AWN-1 serum reacted strongly with the AWN spermadhesins (BHB-7–BHB-9); in the case of AWN-1 protein, the titre was 1:600 000 for 1 µg of the antigen. No reaction was observed with the DQH protein and AQN spermadhesins.

The 14 kDa HNK protein from the BHB-6 fraction did not react with antibodies against either AQN or AWN spermadhesins (Table 2). Only AWN-1 yielded a weak reaction with the non-immune rabbit serum; other protein fractions did not react at all. Immunoreactions of the proteins after transblotting on nitrocellulose sheets were in agreement, but DQH protein reacted with anti-AQN-1 serum with the same intensity as did AQN-1.

Interaction of separated sperm proteins with different ligands

The ligand binding ability of the purified heparin-binding proteins was studied by three methods: (i) detection of proteins separated by SDS-PAGE followed by transfer to nitrocellulose membranes using biotinylated ligands and avidin–peroxidase (the reaction with concanavalin A was also studied using non-derivatized horse radish peroxidase); (ii) interaction of proteins immobilized on microtitre polystyrene plates with biotinylated ligands and avidin–peroxidase in ELBA tests; (iii) affinity chromatography on gelatin–agarose and heparin–polyacrylamide gel followed by phosphoryl choline elution. Results are summarized in Fig. 3 and Tables 2 and 3. All BHB proteins bound to the biotinylated polyacrylamide derivative of heparin but with varying affinity; the strongest interaction was observed in the case of AWN-1 spermadhesin from the BHB-7 fraction. All isolated proteins interacted with concanavalin A indicating their glycoprotein nature. The ELBA tests and electrophoretic detection of the separated proteins showed that the AWN-1 protein had the greatest affinity for solubilized zona pellucida; a weaker interaction was observed in the case of DQH and AQN proteins. The interaction of AWN-2 with zona pellucida was weak, whereas the interaction of DQH, AQN spermadhesins and AWN-1 was stronger (Table 2). In ELBA tests, all boar heparin-binding proteins interacted with zona pellucida. AWN spermadhesins bound to zona pellucida and proteinase inhibitors more strongly than AQN spermadhesins and DQH protein. AWN-1 showed the strongest interaction with zona pellucida and with soya bean proteinase inhibitor (Fig. 3). In all cases, the reactivity was dependent on protein concentration. Affinity chromatography experiments showed that the DQH and AWN proteins but not the AQN proteins bind to gelatin–agarose and that the DQH protein and the dominant AQN-1 spermadhesin are phosphoryl choline-binding

Fig. 3. Binding of different types of ligand by boar heparin-binding proteins BHB-2–BHB-5, BHB-7 and BHB-9 (2.5 µg of each) in an enzyme-linked binding assay. Polyacrylamide derivative of heparin (□, 5 µg); solubilized pig zona pellucida (■, 0.5 µg); trypsin kallikrein inhibitor (▨, 2 µg); soya bean Kunitz trypsin inhibitor (▩, 2 µg).

Table 2. Reaction of blotted boar heparin-binding proteins BHB-2–BHB-9 electrophoretically separated with rabbit antisera to AQN and AWN proteins, biotinylated solubilized pig zona pellucida and concanavalin A

Heparin-binding protein	N-terminal	M_r (kDa)	Reaction with:			
			anti-AQN-1	anti-AWN-1	zona pellucida	concanavalin A
BHB-2	DQH-	13	+++	–	++	+
BHB-3	AQN-	13	+++	–	++	+
BHB-4	AQN-	14	++	–	+++	+
		16	++	–	+	+
BHB-5	AQN-	12	++	–	++	+
BHB-6	HNK-	14	–	–	–	+
	AWN-	16	–	++	+-	+
	AWN-	17	–	++	+-	+
BHB-7	AWN-	14	–	+++	++++	+
BHB-8	AWN-	14	–	++	++	+
	blocked	70	–	–	–	+
BHB-9	blocked	16	–	+++	+	+

+, positive reaction; the number of + indicates the relative immunoreactivity and/or binding to zona pellucida of each protein.
+–, extremely weak pattern.

Table 3. Interaction of separated boar sperm proteins with gelatin and phosphoryl choline as determined by affinity chromatography

Sperm proteins	Gelatin ^a	Phosphoryl choline ^b
DQH	++	++
AQN	–	+ ^c
AWN	++ ^d	–

++, complete binding; +, incomplete binding.

^aProteins bound to gelatin–agarose and eluted with 4 mol urea l⁻¹.

^bProteins bound to heparin–polyacrylamide gel and eluted with phosphoryl choline.

^cAQN-1 protein predominated in the eluted fraction.

^dAll AWN spermadhesins in the eluted fraction.

proteins (Table 3). Individual AQN forms differed in their binding properties.

Binding of boar heparin-binding proteins to epididymal spermatozoa

The ability of the DQH protein to bind to spermatozoa was examined using biotinylated protein and epididymal spermatozoa. Biotinylated DQH protein was found to bind to epididymal spermatozoa immobilized in microtitre plates.

To localize the site of the attachment of the spermadhesin on the sperm cell, epididymal boar spermatozoa were incubated with biotinylated DQH solution and the binding of this protein was detected by FITC-avidin and visualized under the immunofluorescent microscope. The results of this experiment are shown in Fig. 4. The control with non-immune serum was negative (not shown). Figure 4a shows that biotinylated DQH attached to the acrosome of epididymal spermatozoa. Similar results were obtained with spermatozoa treated with biotinylated AQN-1 and AWN-1 (Fig. 4b, c).

Agglutination of splenocytes

The addition of 250 or 100 $\mu\text{g DQH ml}^{-1}$ cell suspension of splenocytes was followed by 80–90% agglutination of splenocytes (Fig. 5). Incubation of the splenocytes with 20 $\mu\text{g DQH ml}^{-1}$ cell suspension agglutinated the cells by 20%. No cell agglutination was observed with 1 $\mu\text{g DQH ml}^{-1}$ splenocyte suspension.

Discussion

In addition to spermadhesins of the AQN and AWN families, the heparin-binding fraction of boar seminal plasma contained HNK and DQH proteins. The HNK protein showed close homology to the dominant basic protein of human seminal plasma (Lilja and Jeppsson, 1985) that is a cleavage product of the human vesicle protein semenogelin I. The enzyme responsible for the cleavage is a prostate kallikrein-like protease (Robert *et al.*, 1997). The presence of a semenogelin-like protein or its degradation product (HNK protein) among boar heparin-binding proteins has not been reported so far. Determination of N-terminal amino acid residues of the DQH protein (BHB-2) showed that this protein is characterized by homology to the main acidic proteins of bull seminal plasma (PDC-109 and BSP-A3) (Seidah *et al.*, 1987) and to HSP-1 and HSP-2 of stallion seminal plasma (Calvete *et al.*, 1995). Affinity chromatography experiments showed that the DQH protein bound to gelatin–agarose, as does bull or stallion major seminal protein (Manjunath *et al.*, 1987; Calvete *et al.*, 1995). The DQH protein also showed phosphoryl choline-binding ability as do the bull major seminal proteins (Calvete *et al.*, 1996). All these proteins isolated in this study also exhibit a very high level of sequence homology with the collagen binding domain of the type II in fibronectin (Bannayi *et al.*,

Fig. 4. Binding of (a) DQH, (b) AQN-1 and (c) AWN-1 proteins from boar seminal plasma to boar epididymal spermatozoa. Scale bars represent 20 μm .

1990). The bull and stallion seminal plasma proteins play an important role in the process of sperm capacitation caused by heparin-like glycosaminoglycans (Miller *et al.*, 1990). DQH protein is also spermadhesin-like because its biotinylated derivative adheres to epididymal spermatozoa *in vitro*. The existence of a boar seminal plasma protein with the N-terminal sequence DQH was first reported by Sanz *et al.* (1993). Our results suggest that the DQH sperm surface protein described here is identical with the adhesion

Fig. 5. Agglutinating activity of DQH sperm surface protein from boar seminal plasma on mouse splenocytes. Scale bar represents 140 μ m.

inducing protein (pAIF-1) isolated from boar seminal vesicle fluid by Hadjisavas *et al.* (1994). pAIF-1 has been implicated in mediating the activation of leucocytes at the site of inflammation in the female reproductive tract that occasionally occurs following insemination at mating (Hadjisavas *et al.*, 1994). In the present study, DQH was found to agglutinate mouse splenocytes. Therefore, it is probable that DQH participates in the inflammatory process. Calvete *et al.* (1997) recently isolated and characterized a heparin- and phosphoryl choline-binding protein from boar and stallion seminal plasma. The primary structure was similar to that of the DQH protein isolated in the present study which has recently been characterized (K. Bezouška, J. Sklenář, P. Novák, P. Halada, V. Havlíček, M. Kraus, M. Tichá, V. Jonáková, unpublished). The ability of DQH to interact strongly with heparin- and chondroitin-like polysaccharides (Tichá *et al.*, 1998) suggests that it also participates in the regulation of sperm capacitation.

A variety of heparin-binding proteins has been isolated from the seminal plasma of different species and similarities have been observed among sperm proteins isolated from different mammalian species. This phenomenon was revealed by N-terminal amino acid sequence homology and immunochemical methods. However, the relative amounts of the different types of sperm protein in the heparin-binding fractions obtained from different species differ significantly (Jonáková, unpublished).

The heparin-binding proteins of boar seminal plasma may be divided into three groups: (i) spermadhesins of the AQN and AWN families; (ii) DQH sperm surface protein; and (iii) HNK protein. While AQN and AWN spermadhesins represent major protein components of boar seminal plasma, homologous proteins found in the seminal plasma of bulls (aSFP; Calvete *et al.*, 1996) and stallions (HSP5; Bellair *et al.*, 1997) are present in smaller amounts. The minor DQH sperm protein of boar seminal plasma is homologous to the major components of bull and stallion seminal plasma (PDC-109, BSP-A3, HSP-1 and HSP-2; Seidah *et al.*, 1987; Calvete *et al.*, 1995). Detection of HNK protein in the heparin-binding fraction of the boar seminal plasma represents one of the few

examples of homologous proteins in boar and human seminal plasma. The human analogue of the HNK protein can be isolated as a major component from the heparin-binding fraction of human seminal plasma (Jonáková, unpublished results).

Calvete *et al.* (1993a,b) reported that only some of the boar spermadhesins are glycoproteins and suggested that the glycosylation of sperm proteins modulated their ligand-binding activity. Our results indicate that all spermadhesins isolated from boar seminal plasma are glycosylated. Amino sugars were detected in all purified heparin-binding fractions and all proteins interacted with concanavalin A (Tichá, unpublished). This discrepancy might be explained by the use of different starting material for the isolation of spermadhesins.

AQN, AWN and DQH proteins bound STI and TKI from bovine organs with different affinities. AQN-3, AWN-1 and AWN-2 bound both inhibitors strongly, the binding ability of AQN-1, AQN-2 and DQH was weak. However, Sanz *et al.* (1992) reported that AQN-1 and AWN, but not AQN-3, bound proteinase inhibitors. We suggest that the weak binding ability of AQN-1 reported here resulted from glycosylation of the protein. Calvete *et al.* (1994) reported that glycosylated spermadhesins bound proteinase inhibitors weakly compared with the strong binding abilities of deglycosylated AWN-1. Spermadhesins that bind proteinase inhibitors strongly may have a role in preventing a premature acrosome reaction.

Immunological properties of isolated protein fractions from boar seminal plasma were studied using polyclonal anti-AQN-1 and anti-AWN-1 antibodies. Two different methods were used: ELISA and immunodetection of electrophoretically separated proteins transferred to nitrocellulose membranes. Anti-AWN antibodies reacted with proteins belonging to the AWN family and anti-AQN antibodies reacted with proteins belonging to the AQN family. Crossreactivity was observed in the DQH protein even though it belongs to a different family of sperm proteins. This unexpected finding suggests that the three-dimensional arrangement of the protein molecules determines their antigenic properties. Determination of N-terminal amino acid sequences should prove that DQH and AQN do not form a complex by aggregation of the protein molecules. The crossreactivity of DQH with anti-AQN 1 could be investigated further by immunological detection of proteins homologous to spermadhesins in tissues and organs of the same animal species or by finding homologous proteins in different animal species. It is likely that the immunological reactions follow a much more complicated molecular mechanism than previously thought.

Our results are in agreement with the current opinion that the proteins located on the sperm surface are involved in the complex chain of events leading to sperm capacitation, gamete recognition and binding to the ovum. Individual sperm proteins that differ in their binding properties might have different roles and participate in the process in different phases and at different locations.

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