Isolation and characterization of heparin- and phosphorylcholine-binding proteins of boar and stallion seminal plasma. Primary structure of porcine pB1

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Received 25 February 1997

Abstract In the bovine, seminal plasma heparin-binding proteins bind to sperm lipids containing the phosphorylcholine group and mediate the capacitating effects of heparin-like glycosaminoglycans during sperm residence in the female genital tract. We report the characterization of heparin- and phosphorylcholine-binding proteins of stallion and boar seminal plasma. Horse seminal plasma proteins HSP-1 and HSP-2, and boar protein pB1, belong to the same family as the bull heparin- and phosphorylcholine-binding proteins BSP-$A_{1/2}$, BSP-A_3, and BSP-30K. We have determined the amino acid sequence and posttranslational modifications of boar glycoprotein pB1. It contains 105 amino acids arranged into a mosaic structure consisting of a N-terminal 18-residue *O*-glycosylated polypeptide followed by two tandemly organized 40–45-residue fibronectin type II domains. pB1 displays 60–65% amino acid sequence similarity with its equine and bovine homologues. However, in their respective seminal plasmas, the BSP and the HSP proteins associate into 90–150-kDa oligomeric complexes, whereas pB1 forms a 35–40-kDa complex with spermadhesin AQN-1. In addition, pB1 appears to be identical to the recently described leukocyte adhesion regulator of porcine seminal fluid pAIF-1. Our results tie in with the hypothesis that homologous proteins from different mammalian species may display distinct biological activities, which may be related to species-specific aspects of sperm physiology.

Key words: Heparin-binding protein; Phosphorylcholine-binding protein; Stallion seminal plasma HSP-1 and HSP-2; Boar seminal plasma pB1 (pAIF-1); Spermadhesin AQN-1; Primary structure; *O*-Glycosylation; Fibronectin type II domain

1. Introduction

Seminal plasma, the fluid in which mammalian spermatozoa are suspended in semen, is made up by the secretions of the male accessory reproductive organs and appears to exert important effects on sperm function [1]. Thus, the seminal plasma of a variety of mammalian species (including pig, cat, rat, goat, horse, ram, bull, rabbit, and human) contains decapacitation factors which prevent inappropriate acrosome reactions [2]. Inactivation or release of these factors might modulate capacitation in vivo during sperm residence in the female's genital tract. On the other hand, bovine seminal plasma contains protein markers associated with Holstein bull fertility [3,4]. In addition, bovine seminal plasma heparin-binding proteins bind to phosphorylcholine-containing lipids of cauda epididymal spermatozoa at ejaculation and invoke sperm capacitation mediated by heparin-like glycosaminoglycans secreted by the epithelium of the female reproductive tract, thereby enhancing the fertilizing capacity of spermatozoa [5]. Furthermore, seminal plasma carbohydrate- and heparin-binding proteins of the spermadhesin family coat the sperm surface of boar and stallion spermatozoa at ejaculation and are thought to participate in sperm capacitation and in the recognition and initial protein-carbohydrate binding mechanism of homologous gametes at fertilization [6,7].

Increasing evidence indicates that the concerted action of positive and negative regulatory seminal plasma factors may modulate the capacitation state and fertilizing capability of mammalian spermatozoa in those species investigated so far. Heparin binding to acceptor molecules of seminal plasma origin which coat the sperm surface at ejaculation has been documented to play a major role in in vitro bull sperm capacitation [5,8,9]. However, both the protein composition of seminal plasma and the physiological effects of heparin vary from species to species [8]. In the horse this effect appears to be moderate; and heparin is not necessary for in vitro sperm capacitation in the pig [8,9]. The primary structures of the major heparin- and phosphorylcholine-binding proteins of bovine seminal plasma (BSP-$A_{1/2}$, BSP-A_3, and BSP-30K) ([9,10] and references therein) show that these proteins belong to the same protein family. Each of these proteins displays a mosaic architecture, which consists of N-terminal distinctly *O*-glycosylated polypeptide extensions of variable length followed by two tandemly arranged, highly conserved fibronectin type II domains. On the other hand, the bulk of the boar seminal plasma heparin-binding proteins are members of the spermadhesin family [11] and do not show phosphorylcholine-binding activity.

Phosphorylcholine-binding proteins from the seminal fluid of different mammalian species share antigenic determinants with bovine BSP-$A_{1/2}$, BSP-A_3, and BSP-30K [12]. Here we report the characterization of stallion and boar seminal plasma heparin- and phosphorylcholine-binding proteins. Our results confirm that the stallion, boar, and bovine phosphorylcholine-binding proteins belong to the same protein family. However, the porcine protein (pB1) displays biological activities other than those documented for bovine and equine phosphorylcholine- and heparin-binding proteins.

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PII S0014-5793(97)00344-X

2. Materials and methods

2.1. Isolation of phosphorylcholine-binding proteins

Boar and stallion seminal plasmas were obtained by 15 min centrifugation at 1500×*g* (room temperature) of freshly ejaculated semen. The supernatant was further clarified by centrifugation at 14000×*g* for 15 min at room temperature, and 100 ml was applied to a heparin-Sepharose CL-6B (Pharmacia) column (16×3 cm) equilibrated in 50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 0.025% (w/v) sodium azide, pH 7.4 (TBS). After washing, the column was eluted with the same buffer containing 10 mM *o*-phosphorylcholine (Sigma). Eluted proteins were dialyzed extensively against TBS and applied to a 10×1 cm *p*-aminophenyl phosphorylcholine (Pierce) column equilibrated in TBS. The column was washed until the absorbance at 280 nm reached baseline, and the bound proteins were eluted with column buffer containing 10 mM phosphorylcholine.

2.2. Characterization of isolated proteins

Protein fractions were analyzed by SDS-(15%) polyacrylamide gel electrophoresis [13]. Boar seminal plasma proteins were separated by reverse-phase HPLC on a Lichrospher RP100 C18 (25×0.4 cm, 5 μm particle size, 100 Å pore size) (Merck) eluted at 1 ml/min with a mixture of 0.1% (v/v) trifluoroacetic acid in water (solution A) and acetonitrile (solution B), first isocratically (25% B) for 5 min, followed by a gradient of 25–35% B for 10 min, and 35–50% for 60 min. Stallion seminal plasma proteins were separated using the same procedure as for the boar proteins but using the chromatographic conditions described in [14]. Detection was done at 220 nm, peaks were collected manually, and dried using a Speed-Vac (Savant).

N-terminal amino acid sequence analyses were done with Applied Biosystems sequencers 473A and Procise following the manufacturer's instructions.

Molecular masses of reverse-phase HPLC isolates were determined by electrospray ionization mass spectrometry using a Sciex API-III triple quadrupole mass spectrometer.

Apparent molecular masses of heparin-bound, phosphorylcholine-eluted protein mixtures were determined by size exclusion chromatography using a Superose-12 column and a FPLC system. The column was equilibrated in 10 mM MOPS, pH 7.0, with or without 10 mM phosphorylcholine.

Quantification of the protein content in the chromatographic fractions and amino acid composition of isolated proteins were carried out using an Alpha Plus amino acid analyzer (Pharmacia) after sample hydrolysis with 6 M HCl for 24 h at 110°C in evacuated and sealed ampoules.

For amino sugar and neutral sugar analyses, the samples were hydrolyzed with 4 M HCl for 4 h or 2 M HCl for 2 h at 110°C. For sialic acid determination, the samples were hydrolyzed with 0.2 M trifluoroacetic acid for 1 h at 80°C. After drying the hydrolysates in a SpeedVac, the monosaccharides were resolved on a CarboPac PA1 column (25×0.4 cm) eluting at 1 ml/min isocratically with either 16 mM NaOH (for amino and neutral sugars) or 20 mM NaOH in 60 mM sodium acetate (for sialic acid), and analyzed using a Dionex DX-300 analyzer equipped with pulsed amperometric detector and the AI-450 chromatography software [15].

Analytical ultracentrifugation was done in a Beckman XL-A centrifuge with absorption optics. Sedimentation velocity runs were performed in standard double sector cells. Molecular masses were evaluated from sedimentation velocity experiments by simultaneous determination of the sedimentation and diffusion coefficients from the movement and broadening of the sedimenting band according to Lamm's equation [16] as described [17].

2.3. Peptide mapping

For peptide mapping, isolated proteins (2–5 mg in 100 mM ammonium bicarbonate, pH 8.3) were digested with trypsin (Sigma) or endoproteinase Lys-C (Boehringer Mannheim) at an enzyme:substrate ratio of 1:100 (w/w) for 18 h at 37°C. Proteolytic peptides were separated by reverse-phase HPLC on a Lichrospher RP100 C18 25×0.4 cm column (Merck) (5 μm particle size) eluted at 1 ml/min with a gradient of 0.1% (v/v) trifluoroacetic acid in (solution A) water and (solution B) acetonitrile as follows: isocratically with 10% solution B for 5 min, followed by 10–35% solution B for 105 min, and 50–70% solution B for 30 min. Cleavage of proteins (10 mg/ml in 70% (v/v) formic acid) at methionine residues was performed with cyanogen bromide (100 mg/ml) overnight at room temperature, in the dark, and under a nitrogen atmosphere. CNBr-derived fragments were isolated by reverse-phase HPLC as above using the following chromatographic conditions: isocratically (10% B) for 5 min, followed by 10–50% B for 80 min, and 50–70% B for 20 min.

2.4. Titration of thiol groups and location of disulfide bonds

For quantitation of free cysteine residues and disulfide bonds the protein (2 mg/ml in 100 mM Tris-HCl, pH 8.6, 1 mM EDTA, 6 M guanidine hydrochloride) was incubated with either 10 mM iodoacetamide for 1 h at room temperature, or with 1% (v/v) 2-mercaptoethanol for 2 min at 100°C followed by addition of a 2.5 molar excess of iodoacetamide over reducing agent for 1 h at room temperature. Both samples were dialysed against deionized (MilliQ) water, lyophilized, and subjected to amino acid analysis. For location of cystine residues, native pB1 was degraded with trypsin and the peptides were isolated by reverse-phase HPLC (as above) and characterized by amino acid analysis.

2.5. Similarity search and prediction of O-glycosylation sites

Amino acid sequence similarity searches were carried out using the program FASTA [18] implemented in the GCG Sequence Analysis Software Package and the protein sequences deposited in the data bank of the Martinsried Institute for Protein Sequences (MIPS). The pB1 amino acid sequence was submitted to the NetOGlyc server [19] for prediction of *O*-glycosylation sites [20].

3. Results

3.1. Isolation of stallion and boar phosphorylcholine-binding proteins

For isolation of heparin- and phosphorylcholine-binding proteins from the stallion and the boar, the seminal plasmas were subjected to affinity chromatography on heparin-Sepharose and bound proteins were eluted with phosphorylcholine. This method has been employed for fractionation of bovine seminal plasma proteins [21]. In both cases, the stallion and boar proteins recovered in the phosphorylcholine eluates were resolved into two protein fractions by reverse-phase HPLC (Fig. 1). N-terminal sequence determination, peptide mapping, and compositional and mass spectrometric analyses showed the stallion proteins to be HSP-1 and HSP-2 (peaks 1 and 2 in Fig. 1A, respectively), which had been previously characterized as the major heparin- and gelatin-binding glycoproteins of equine seminal plasma [14,22]. Both proteins are structurally related and belong to the same protein family as the bovine phosphorylcholine-binding proteins BSP-$A_{1/2}$, BSP-A_3, and BSP-30K ([10] and references therein). At positions 67–69 of HSP-1 we found SWK instead of IQV, as previously reported [22]. Sequence analyses of several cDNA

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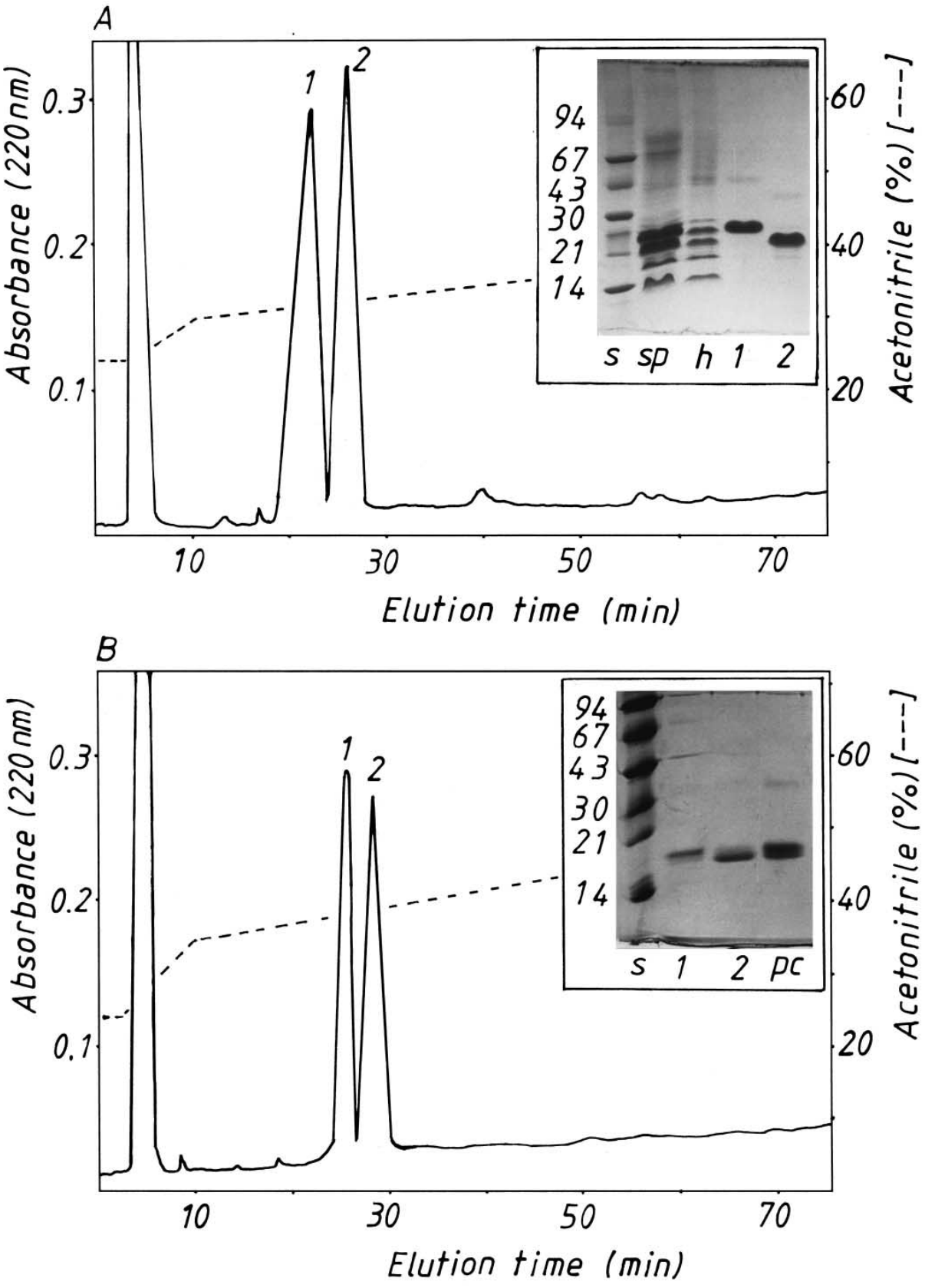
Fig. 1. Characterization of phosphorylcholine-binding proteins. Reverse-phase HPLC separation of heparin-bound proteins from stallion (A) and boar (B) seminal plasma proteins eluted with phosphorylcholine. Inserts, SDS-gel polyacrylamide electrophoretic analysis of protein fractions: (A), sp, total seminal plasma proteins; h, proteins bound to the heparin-Sepharose column eluted with 1 M NaCl; 1 and 2, peaks 1 and 2 obtained by reverse-phase separation of the subset of heparin-bound proteins eluted with phosphorylcholine. (B) Lanes 1 and 2, peaks 1 and 2 obtained by reverse-phase separation of the subset of heparin-bound proteins eluted with phosphorylcholine; pc, total proteins eluted from the heparin-Sepharose column with phosphorylcholine. In A and B, lane s show molecular mass markers: from top to bottom, phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (21 kDa), and lysozyme (14 kDa).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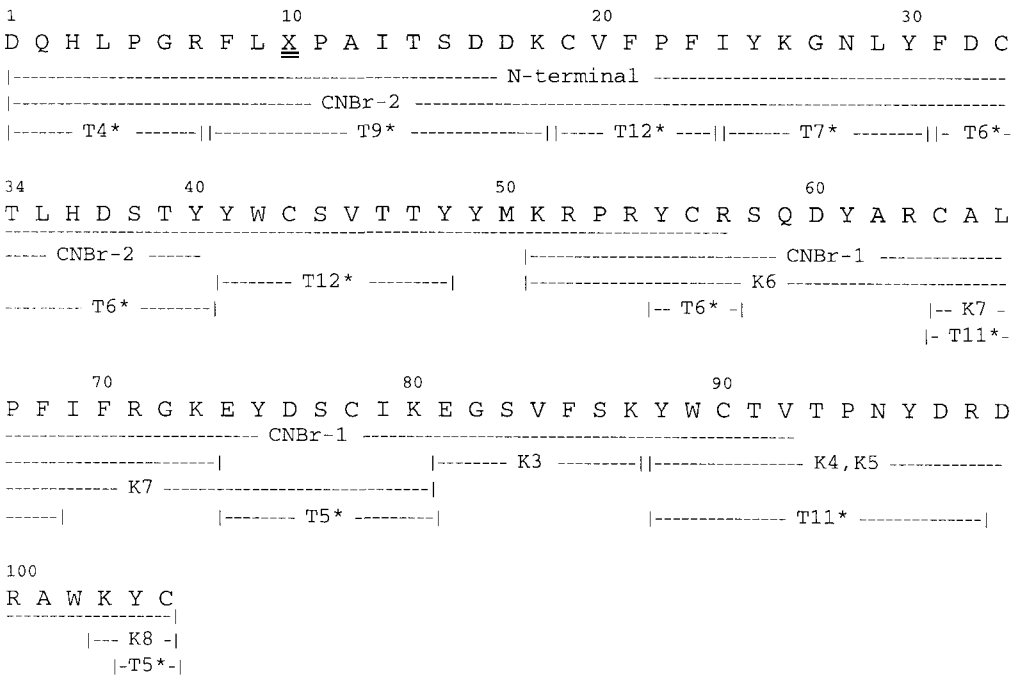

Fig. 2. The primary structure of boar pB1. Fragments labeled CNBr and K are peptides obtained by degradation of pyridylethylated pB1 with cyanogen and endoproteinase Lys-C, respectively. T* denotes tryptic peptides of native pB1; X is an unidentified residue, which correspond to an *O*-glycosylated threonine.

clones confirmed that HSP-1 actually contains the sequence SKW (S. Bellair et al., unpublished results).

On the other hand, the N-terminal sequences of pyridylethylated boar proteins were: DQHLPGRFLXPAITSDDKCVFPFIYKGNLYFDCTLHDSTYYWCSVTTYYMKR and AQNKGPHKCGGVLRDLSGRISTYEGPKTDCIWTILAKPGS (Fig. 1B, peaks 1 and 2, respectively). The N-terminal sequence of peak 1 has been previously reported for a minor boar seminal plasma heparin-binding protein termed pB1 [11] and for a leukocyte adhesion stimulating factor isolated from porcine seminal fluid [23]. The N-terminal sequence of peak 2 together with the molecular mass of the protein (which was 11 828 ± 1 Da) identified this protein as spermadhesin AQN-1 [24].

Quantitation of proteins, done by amino acid analysis after reverse-phase HPLC separation, showed that in both cases, HSP-1 and HSP-2, and pB1 and AQN-1 were present in roughly equimolar concentrations in the stallion and boar heparin-bound, phosphorylcholine-eluted fractions, respectively.

When subjected to affinity chromatography on phosphorylcholine-agarose, HSP-1 and HSP-2 bound to the affinity column both as a mixture and when HPLC-isolated proteins were analyzed. However, when either the pB1/AQN-1 mixture or the individual components (separated by reverse-phase HPLC) were chromatographed, only pB1 bound to the phosphorylcholine-agarose column.

The apparent molecular mass of the HSP-1/HSP-2 mixture, determined by size exclusion chromatography after removal of phosphorylcholine by extensive dialysis, was 90 kDa. The same value has been previously reported for heparin-bound HSP-1/HSP-2 mixture eluted with 1 M NaCl [25]. However, when the 90-kDa HSP-1/HSP-2 complex was chromatographed in buffers containing 20 mM phosphorylcholine, an asymmetric double peak with apparent molecular masses of 38 and 25 kDa was obtained. Reverse-phase HPLC analysis revealed that HSP-1 and HSP-2 were quantitatively distributed in the 38-kDa and 25-kDa FPLC peaks, respectively.

pB1 and AQN-1, in the absence of phosphorylcholine, eluted together in a size exclusion chromatographic fraction with apparent molecular mass of 35–40 kDa. Analytical ultracentrifugation of pB1 and AQN-1, separated by affinity chromatography on phosphorylcholine-agarose, showed that these proteins sedimented with 1.7 S and 2.9 S, respectively, which correspond to a monomeric (14 kDa) and a dimeric (23 kDa) protein, respectively.

3.2. The primary structure of pB1

The amino acid sequence of boar pB1 was established from structural information gathered by N-terminal sequencing of the intact protein and degradation peptides obtained by deg-

Table 1
Amino acid of heparin- and phosphorylcholine-binding proteins porcine pB1, equine HSP-1, and bovine PDC-109 and BSP-30K containing *O*-glycosylated threonine residues (X)

Protein	Sequence	Score
pB1	G R L F X P A I T S D	0.297
HSP-1	D L Q T X G A D H S A	0.543
	D H S A X V N P D Q Q	0.557
	Q L I M X K H S A T V	0.438
	K H S A X V T P E N K	0.366
PDC-109	S T E P X Q D P A E L	0.149
BSP-30K	G S K P X P S G M A D	0.665
	D E L P X E T Y D L P	0.495
	P E I Y X T T F L P R	0.350
	E I Y T X T F L P R T	0.174
	I Y T T X F L P R T I	0.305
	F L P R X I Y P Q E E	0.665

Score indicates the prediction strength for threonine: a value above the threshold of 0.5 denotes a high probability of becoming *O*-glycosylated.

Fig. 3. Boar, bovine, and stallion seminal plasma phosphorylcholine-binding proteins. Alignment of the amino acid sequence of boar pB1 with those of bovine BSP-30K, PDC-109 [14] and BSP-A_3 [15] and equine HSP-1. The occurrence of unique short repeats (denominated I, II, and III) at the N-terminal part of BSP-30K, and the N-terminal extension (labeled A) of HSP-1 are shown. X, *O*-glycosylated threonine residue. Fn II-a and Fn II-b, conserved fibronectin type II domains.

radation of pyridylethylated pB1 with cyanogen bromide and endoproteinase Lys-C (Fig. 2). The protein has 105 amino acids. Quantitation of sulfhydryl groups and disulfide bonds clearly showed that the 8 cysteine residues of pB1 are engaged in four disulfide bridges. They were located by characterization of tryptic peptides obtained from digestion of the native protein: disulfide bonds between C^{19}-C^{43}, C^{33}-C^{56}, C^{64}-C^{78}, and C^{90}-C^{105} were found in peptides T12*, T6*, T11*, and T5*, respectively (Fig. 2).

Carbohydrate analysis showed that pB1 contains *N*-acetylgalactosamine, galactose, and *N*-acetylneuraminic acid, indicating that pB1 is an *O*-glycosylated protein. The residue at position 10 could not be identified and carbohydrate was found only in fragments CNBr-2 and T9* (Fig. 2), strongly suggesting that this position carries the single *O*-glycosylation site of pB1. In addition, amino acid analysis of peptide T9* demonstrated that the glycosylated residue is threonine.

The amino acid sequence of pB1 displays 60–65% similarity with stallion HSP-1 and bovine BSP-$A_{1/2}$ (also called PDC-109), BSP-A_3, and BSP-30K (Fig. 3). Like the stallion and bovine proteins, pB1 displays a mosaic structure built by a short (18 residues) *O*-glycosylated N-terminal polypeptide followed by a tandem of fibronectin type II (Fn II) modules. The amino acid sequence similarity is mainly restricted to the C-terminal Fn II domains (Fig. 3).

The fact that bovine PDC-109, stallion HSP-1 and HSP-2, and boar pB1 are *O*-glycosylated on threonine residues suggests a common mechanism of glycosylation by a UDP-*N*-acetyl-D-galactosamine: threonine-*N*-acetyl-galactosaminyltransferase in the three species. In Table 1 the amino acid sequences containing *O*-glycosylated threonine residues of the four glycoproteins are compared along with the score for threonine glycosylation assign by the NetOGlyc server [19]. The absence of structural characteristics common to the 12 amino acid sequences (Table 1) and the different scores obtained indicate that the galactosyltransferase may display few amino acid sequence requirements and may glycosylate suitably exposed threonine residues.

4. Discussion

Seminal plasma phosphorylcholine-binding proteins, which share antigenic determinants with bovine BSP-$A_{1/2}$, BSP-A_3, and BSP-30K have been identified in a number of species including bull, human, boar, hamster, mouse, and rat [12]. Here we have isolated and characterized the heparin- and phosphorylcholine-binding proteins of stallion and boar seminal plasmas (HSP-1 and HSP-2, and pB1, respectively). These proteins are structurally related and belong to the same protein family as the bovine BSP proteins.

Our results indicate that stallion seminal plasma HSP-1 and HSP-2 associate into a 90-kDa hetero-oligomer with both subunits displaying heparin- and phosphorylcholine-binding activity. This oligomer is dissociated by phosphorylcholine into HSP-1 and HSP-2 monomers (apparent molecular mass by SDS-PAGE 28 and 22 kDa, respectively). From these data we conclude that the 90-kDa HSP-1/HSP-2 oligomer corresponds most probably to a tetrameric assembly of HSP-1 and HSP-2. On the other hand, boar seminal plasma pB1 and AQN-1 form a stoichiometric 35–40-kDa complex, which possesses phosphorylcholine-binding activity associated with the pB1 subunit. From its apparent molecular mass, we propose

that the pB1/AQN-1 complex might have the composition $(pB1)_2(AQN\text{-}1)_2$. This oligomer is also dissociated through interaction with phosphorylcholine into monomeric pB1 and AQN-1 dimers.

In bull seminal plasma, the major phosphorylcholine- and heparin-binding protein PDC-109 exists as a 90–120-kDa homo-oligomer [26], which can be dissociated to homodimer by interaction with phosphorylcholine (data not shown). Hence, it seems that the phosphorylcholine- and heparin-binding proteins from bull, stallion, and boar seminal plasmas share the property of forming phosphorylcholine-dependent oligomeric structures.

Here, and in previous works [10,22,27,28], we have shown that the major bovine, stallion, and boar seminal plasma heparin- and phosphorylcholine-binding proteins display a modular architecture composed of two tandemly arranged and largely conserved C-terminal fibronectin type II domains and unique N-terminal extensions of variable length that are *O*-glycosylated to different extents. Although it remains to be established whether the variable N-terminal extensions or the conserved Fn II domains are responsible for the oligomerization activity, the observations that the type and extent of glycosylation indirectly affects the heparin-binding capability of stallion seminal plasma HSP-1 and HSP-2 proteins through modulation of their aggregation state [22,25] points to the variable N-terminal polypeptides as the oligomerization-determining domain.

The bovine proteins have been implicated in sperm capacitation modulated by heparin [5,29,30], suggesting that the stallion and boar homologues could play a similar physiological role. The effect of heparin in these mammalian species differs, however, from that observed in the bovine. Thus, heparin-like glycosaminoglycans (GAGs) of follicular fluid exert a moderate effect on stallion sperm capacitation [31] and act as a acrosome-reaction-inducing factor in the pig [32]. Indeed various authors have posed the question of whether GAG action mechanisms in prefertilization processes might be species-specific [33]. We hypothesize that formation of different oligomers may determine the distinct biological effects exerted by seminal plasma heparin-binding proteins in bovine, equine, and porcine species.

Finally, Hadjisavas et al. [23] have characterized a leukocyte adhesion-inducing 15-kDa protein (pAIF-I) from porcine seminal vesicle fluid which has the same 32 N-terminal amino acids as pB1. pAIF-I is proposed to mediate activation of leukocytes at the site of inflammation which occurs occasionally upon mating. This suggests that seminal plasma and sperm-associated pB1 molecules may play diverse biological roles. Whether the same holds for homologous proteins from other mammalian species deserves further investigation.

Acknowledgements: This work has been financed by grants from the Deutsche Forschungsgemeinschaft (Ca 209/1-1) and the Bundesministerium für Forschung, Bildung, und Technologie (01KY9503), Bonn, Germany, and from the Dirección General de Investigación Científica y Técnica (PB95-0077), Madrid, Spain.

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