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Effect of glycosylation on the heparin-binding capability of boar and stallion seminal plasma proteins

Juan J. Calvete[a,b,*], Markus Reinert[a], Libia Sanz[a], Edda Töpfer-Petersen[a]

[a]*Institut für Reproduktionsmedizin, Tierärztliche Hochschule Hannover, Bünteweg 15, 30559 Hannover-Kirchrode, Germany*

[b]*Instituto de Química-Física Rocasolano CSIC, c/ Serrano 119, 28006 Madrid, Spain*

Abstract

Boar and stallion seminal plasmas were fractionated using affinity chromatography on heparin-Sepharose. In both species, among other proteins, the heparin-binding (H^+) and non-heparin-binding (H^-) fractions each contained glycoforms of either porcine PSP-I or equine HSP-1 and HSP-2. However, porcine H^+/PSP-I eluted as a monomeric protein, whereas H^-/PSP-I formed a heterodimer with PSP-II, another major seminal plasma protein. On the other hand, the stallion proteins H^+/HSP-1 and H^+/HSP-2 eluted together as an aggregate of relative molecular mass (M_r) 90 000, whereas H^-/HSP-1 and H^-/HSP-2 eluted as monomers (15 000). Remarkably, when PSP-I and PSP-II from the H^- fraction were separated, both proteins bound to heparin. Altogether these data show that glycosylation has an indirect effect on the heparin-binding ability of PSP-I, HSP-1 and HSP-2 through modulation of their aggregation state.

1. Introduction

In many mammalian species, sperm capacitation in the female's genital tract, a complex and poorly understood process, prepares ejaculated spermatozoa to undergo the acrosome reaction in response to interaction with its homologous zona pellucida, the oocyte's extracellular glycoprotein network [1]. Proteoglycans with heparin and chondroitin sulphate-like glycosaminoglycan side-chains are secreted by the epithelium of the female reproductive tract, particularly at high concentrations during the follicular phase of the estrous cycle, and have been shown to specifically invoke sperm capacitation in a number of mammalian species, as measured by the onset of agonist-inducible acrosome reactions (Ref. [2] and references therein). Thus, in vitro incubation of epididymal sperm from different mammalian species, as bovine, hamster, human, rabbit or equine, with glycosaminoglycans significantly accelerated the development of the sperm-capacitated state as judged by their increased ability to fertilize homologous eggs. In addition, the fact that exposure of bovine epididymal spermatozoa to seminal plasma from the same species enhanced their ability to undergo the acrosome reaction in the presence of heparin [3] indicated that the effects of heparin-like glycosaminoglycans are mediated by seminal plasma heparin-binding proteins which become coated to the sperm surface at ejaculation [4,5].

Seminal plasma heparin-binding proteins have been structurally characterized in only few mammalian species, particularly bovine, pig and horse. The primary structures of the bovine

* Corresponding author.

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seminal plasma 15 000–17 000 relative molecular mass heparin-binding proteins, designated BSP-A_1/A_2 (PDC-109) and BSP-A_3, and the porcine seminal plasma heparin-binding proteins AQN-1, AQN-2, AQN-3, AWN-1 and AWN-2 have been reported [6–12]. The bovine and the porcine proteins are structurally unrelated, but in each species they belong to the same protein family; the bovine proteins are made up of two fibronectin type-II domains [6,7], and the porcine proteins are members of the spermadhesin family [8–12]. The heparin-binding proteins of stallion seminal plasma have recently been isolated and characterized [13,14]. This species contains proteins which are structurally related to both the bovine and the porcine protein families (termed HSP-1, HSP-2, and AWN, respectively). This suggests that the structure and relative abundance of members of different heparin-binding protein families in seminal plasma of different species may be related to the species-specific modulation of sperm capacitation–acrosome reaction exert by heparin. However, although the structure and binding properties of the members of the porcine and bovine seminal plasma heparin-binding protein families have been extensively studied [15–24], the molecular mechanism of heparin-mediated development of the sperm-capacitated state remains largely to be elucidated. Here we show that the degree and/or the type of glycosylation determine the aggregation state of the porcine (PSP-I) and equine (HSP-1 and HSP-2) seminal plasma proteins, and that this, in turn, indirectly modulates the heparin-binding activity of these glycoproteins.

2. Experimental

Boar and stallion ejaculates were collected from healthy reproductively active animals by means of an artificial vagina. Seminal plasma was separated from spermatozoa by centrifugation at 12 000 *g* for 10 min at room temperature using a Biofuge 13 centrifuge (Heareus Sepatech, Jürgens, Hannover, Germany). Seminal plasma proteins were then fractionated and characterized following the steps shown in Fig. 1, which are described below in detail.

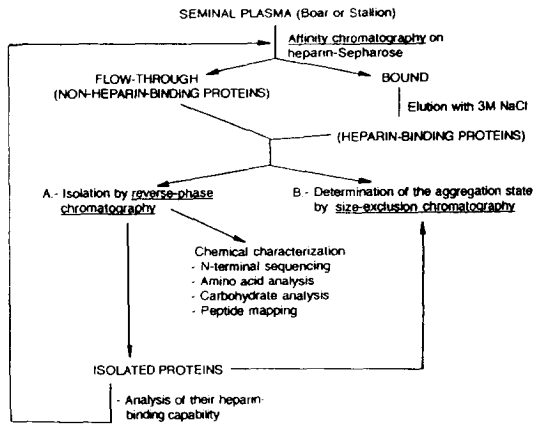

Fig. 1. Diagram of the overall strategy employed for the isolation and characterization of heparin-binding and non-heparin-binding isoforms of seminal plasma proteins of boar and stallion.

Heparin-binding proteins of boar and stallion seminal plasmas were isolated by affinity chromatography using a heparin-Sepharose CL-6B column (Pharmacia Biotech, Freiburg, Germany) equilibrated with 20 m*M* phosphate, pH 7.4. Elution of bound proteins was performed with the same buffer containing 3 *M* NaCl [25].

Isolation of the proteins in the flow-through and the bound fractions was done by reversed-phase high-performance liquid chromatography (RP-HPLC) using a LiChrospher RP-100 C_{18} column (25 × 4 mm, 5 μm particle size) (Merck, Darmstadt, Germany) eluted at 1 ml/min with 0.1% trifluoroacetic acid in water (solution A) and acetonitrile (solution B) first isocratically with 25% B for 5 min, followed by a gradient of 25–30% B for 5 min and 30–70% B for 160 min.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was done according to Ref. [26].

N-Terminal amino acid sequence analyses were carried out with an Applied Biosystems Model 473A (Foster City, CA, USA) automated sequencer, following the manufacturer's instructions.

Amino acid analyses were carried out using an Alpha Plus (Pharmacia Biotech) amino acid

analyzer after sample hydrolysis at 110°C with 6 *M* HCl for 24 h in sealed ampoules.

For amino sugar and neutral sugar analyses, the samples were hydrolyzed at 110°C with 4 *M* HCl for 4 h or 2 *M* HCl for 2 h, respectively. Sialic acid determination was done after hydrolysis at 80°C with 0.2 *M* trifluoroacetic acid. The monosaccharides were resolved on a CarboPac PA1 column (250 × 4 mm) eluting at 1 ml/min isocratically with either 16 m*M* NaOH (amino and neutral sugars) or 20 m*M* NaOH in 60 m*M* sodium acetate, and analyzed using a Dionex DX-300 carbohydrate analyzer (Dionex, Idstein, Germany) equipped with a pulsed amperometric detector and the AI-450 chromatographic software [27].

For peptide mapping, the isolated proteins (2–5 mg/ml in 100 m*M* Tris–HCl, 1 *M* guanidine hydrochloride, pH 8.6) were digested with TPCK-trypsin (Sigma, Munich, Germany) at an enzyme/substrate ratio of 1:100 (w/w) for 18 h at 37°C. Tryptic peptides were isolated by RP-HPLC using the same chromatographic system as above and the following elution condition: first isocratic with 10% B for 5 min followed by a gradient of 5–50% B in 90 min.

Size-exclusion chromatography was carried out using an FPLC system (Pharmacia Biotech, Freiburg, Germany) with a Superose-12 column equilibrated with 50 m*M* Tris–HCl, 150 m*M* NaCl, 0.025% sodium azide, pH 7.4, and eluted at 0.30 ml/min. A mixture of bovine serum albumin (M_r 67 000), ovalbumin (M_r 43 000) and lysozyme (M_r 14 000) was used as a standard for calibrating the column. The proteins in the different fractions were isolated by RP-HPLC and characterized as above.

3. Results

The heparin-binding proteins of boar and stallion seminal plasmas were isolated by affinity chromatography on heparin-Sepharose, and characterized following the steps shown in Fig. 1. Analysis of the protein composition in the flow-through and the bound fractions was done using RP-HPLC (Figs. 2 and 3). It revealed that each

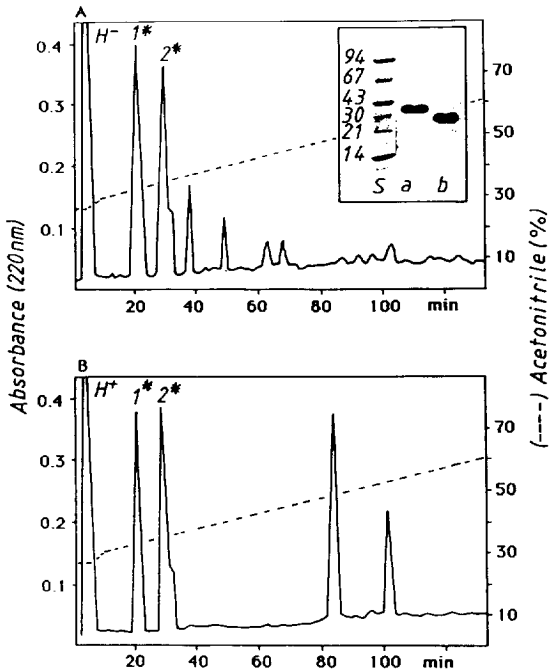

Fig. 2. Comparison of the protein patterns of (A) the non-heparin-binding (H^-) and (B) the heparin-binding (H^+) fractions of stallion seminal plasma using RP-HPLC. The peaks labelled 1* and 2* contained apparently identical proteins in both fractions. The inset in panel A shows 10% SDS-PAGE of isolated proteins 1* (lane a) and 2* (lane b) from the H^- fraction. Identical result was obtained when the corresponding proteins of the H^+ fraction were analyzed. Lane S, standard molecular mass markers; from top to bottom, phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme, whose molecular masses in kDa are shown at the left.

species contained both different and apparently identical proteins (labelled with asterisks in Figs. 2 and 3) in the flow-through and the bound fractions. SDS-PAGE showed that chromatographic peaks eluting at identical position contained proteins with the same apparent molecular masses, which were 35 000 and 25 000 in the case of the horse seminal plasma (HSP) proteins 1 and 2 (inset in Fig. 2A), respectively, and 15 000–20 000 for the boar protein (inset in Fig. 3B). In addition, amino acid and N-terminal amino acid sequence analyses and tryptic peptide mapping (data not shown) showed that the corresponding stallion and boar proteins in the non-heparin-binding (H^-) and the heparin-bind-

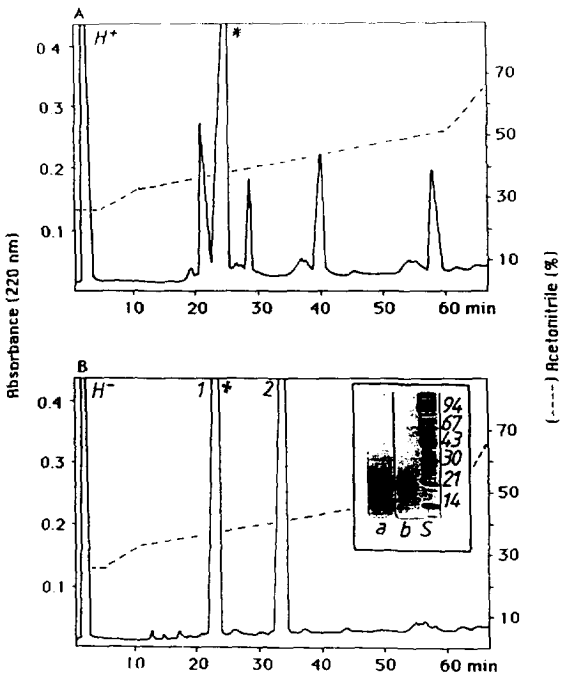

Fig. 3. Characterization by RP-HPLC of the protein patterns of (A) the heparin-binding (H^+) and (B) the non-heparin-binding (H^-) fractions of boar seminal plasma. The peaks labelled with an asterisk contained apparently identical proteins as judged by amino acid analysis, N-terminal sequencing and peptide mapping. The inset in panel B shows the electrophoretic analysis of proteins 1* (lane a) and 2 (lane b) from the H^- fraction. Lane S, standard molecular mass markers (see legend of Fig. 1).

ing (H^+) fractions were structurally indistinguishable within each species, and that the amino acid sequences of these proteins were identical to stallion HSP-1, HSP-2 and boar PSP-I, respectively [9,12–14] (Fig. 4).

Next, we sought to investigate whether in each species (a) homologous non-heparin-binding and heparin-binding proteins are differently post-translational modified, or (b) their heparin-binding activity could be modulated by alteration of their aggregation state.

Carbohydrate analyses of isolated tryptic peptides showed that stallion HSP-1 and HSP-2 contained four and two O-glycosylation sites, respectively (underlined X in Fig. 4), and boar PSP-I contained a single N-linked oligosaccharide attached to asparagine-47 (Fig. 4). The

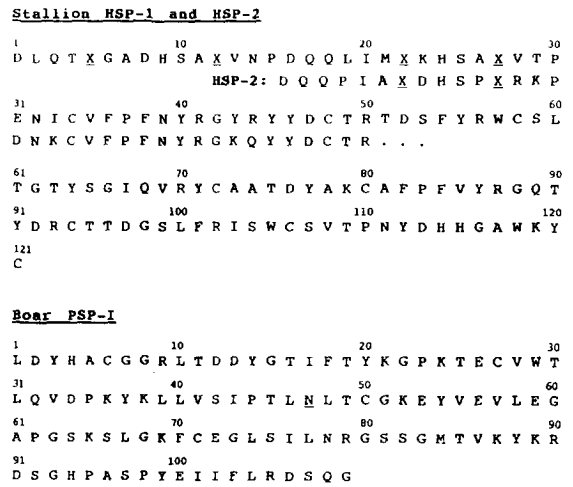

Fig. 4. Complete primary structures of stallion HSP-1 [12] and boar PSP-I [9,10]. The amino acid sequence of HSP-2 [7] has not been completed. X, O-glycosylated threonine residues. The glycosylated asparagine residue at position 47 of PSP-I is underlined.

number and position of these glycosylation sites was conserved in the homologous heparin-binding and non-heparin-binding isoforms of both species. However, whereas the H^+ and H^- HSP-2 proteins contained similar carbohydrate composition, H^+/HSP-1 and H^-/HSP-1 as well as H^+/PSP-I and H^-/PSP-I differed in their carbohydrate content (Table 1).

Then, we examined the aggregation state of HSP-1, HSP-2 and PSP-I in the affinity chromatography fractions. When the stallion and boar seminal plasma heparin-binding and non-heparin-binding fractions were analyzed by size-exclusion chromatography, it was found that H^+/

Table 1
Carbohydrate composition (mol/mol of protein) of the heparing-binding (H^+) and non-heparin-binding (H^-) stallion HSP-1 and HSP-2 and boar PSP-I isoforms

Protein	GlcNAc	GalNAc	Man	Gal	Fuc	NAcNA
H^+/HSP-1	–	3.6	–	3.8	–	3.8
H^-/HSP-1	–	3.2	–	0.9	–	0.2
H^+/HSP-2	–	1.9	–	1.6	–	1.6
H^-/HSP-2	–	1.8	–	1.2	–	2.0
H^+/PSP-I	1.8	–	2.9	–	–	–
H^-/PSP-I	3.6	–	2.5	1.4	0.6	1.2

HSP-1 and H^+/HSP-2 as well as H^-/HSP-1 and H^-/HSP-2 eluted in each case together with apparent molecular masses of 90 000 and 15 000, respectively (Fig. 5A, peaks labelled with asterisks). In both fractions, HSP-1 and HSP-2 were the major components. This indicated that the aggregation state of the heparin-binding proteins is a multimer, whereas the non-heparin-binding HSP isoforms are monomeric species. On the other hand, whereas porcine H^+/PSP-I eluted as a monomeric protein (M_r 14 000), H^-/PSP-I was recovered in the same M_r 30 000 fraction as PSP-II (Fig. 5B, peaks labelled with asterisks), the other major component in the porcine seminal plasma non-heparin-binding fraction (Fig. 3). Although it remains to be elucidated whether the oligomeric complexes of H^+/HSP (proteins 1 and 2) and H^-/PSP-I and H^-/PSP-II are actually homo- or heterocomplexes, initial quantification of the individual proteins along the chromatographic peaks show that in both cases the complexes contain equimolar amounts of the individual proteins, suggesting that H^+/HSP (proteins 1 and 2), and H^-/PSP-I and H^-/PSP-II form most probably heteromultimers and heterodimers, respectively.

To ascertain the effect of glycosylation on the heparin-binding ability of isolated HSP-1, HSP-2, PSP-I and PSP-II, these proteins were purified from the non-heparin-binding fraction of stallion and boar seminal plasmas, respectively, using RP-HPLC, dialyzed against 20 m*M* phosphate, pH 7.4, and subjected to either affinity chromatography on heparin-Sepharose or size-exclusion chromatography (Fig. 1). It was found that none of the stallion proteins were active while both PSP-I and PSP-II possessed the ligand-binding activity (data not shown). On the other hand, all four proteins behave as monomeric proteins on gel-filtration chromatography.

4. Discussion

Analysis of the non-heparin-binding (H^-) and the heparin-binding (H^+) fractions of boar and stallion seminal plasmas showed that each fraction contained glycoforms of porcine PSP-I and equine HSP-1 and HSP-2, respectively. The compositional data shown in Table 1 suggest that H^+/HSP-1 may contain four sialylated (tri)saccharides (possible structure: NAcNA-Gal-Gal-NAc-) whereas H^-/HSP-1 contains non-sialylated structures, i.e. one disaccharide Gal-GalNAc- and three single GalNAc- oligosaccharides. On the other hand, the carbohydrate

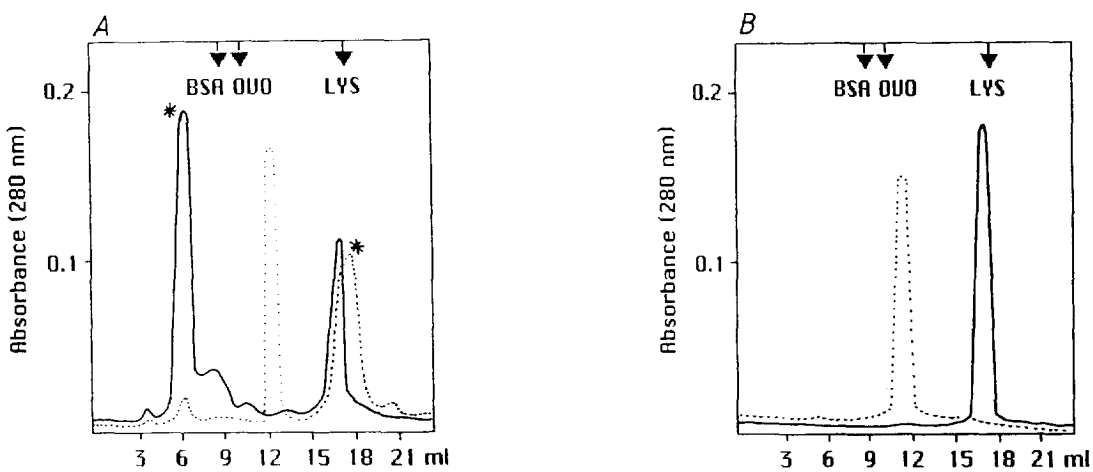

Fig. 5. Size-exclusion chromatographic separation of the stallion (A) and boar (B) seminal plasma proteins of the heparin-binding (solid line) and non-heparin-binding (dashed line) fractions. *, chromatographic fractions containing HSP-1 and HSP-2. The elution positions of molecular mass standards bovine serum albumin (BSA, M_r 67 000), ovalbumin (OVO, M_r 43 000) and lysozyme (LYS, M_r 14 000) are shown.

composition of H^+/PSP-I resembles that of oligomannose structures, whereas the sugar content of H^-/PSP-I may correspond to sialylated biantennary complex-type oligosaccharides. The presence of oligomannose carbohydrate chains in H^+/PSP-I is in agreement with previously reported lectin mapping results [9].

The finding of different glycoforms in the non-heparin-binding and the heparin-binding fractions raised the question of whether glycosylation was the primary determinant of the different biological activity of the H^+ and H^- HSP-1, HSP-2 and PSP-I proteins.

Examination by size-exclusion chromatography of the aggregation state of PSP-I, HSP-1 and HSP-2 in the flow-through and bound heparin-affinity chromatography fractions indicated that glycosylation might not directly affect the heparin-binding capability of the stallion and porcine seminal plasma proteins, but might rather determine the aggregation state of the glycoproteins. Thus, the facts that (a) porcine PSP-I is a monomeric heparin-binding protein but forms a non-heparin-binding heterodimer with PSP-II, (b) both PSP-I and PSP-II when isolated by RP-HPLC from the non-heparin-binding fraction eluted as monomeric proteins by gel-filtration chromatography and possessed heparin-binding capability, and (c) stallion heparin-binding HSP-1 and HSP-2 associated into an M_r 90 000 complex whereas both proteins were monomeric in the non-heparin-binding fraction, further supported our hypothesis that the aggregation state of the glycoproteins is the primary determinant for expression of their heparin-binding capability.

Our results evidence that the seminal plasma heparin-binding proteins from two mammalian species, which belong to different protein families, have different structural requirement for expression of heparin-binding activity. Thus, whereas in the case of horse seminal plasma proteins multimer formation seems to be a prerequisite for heparin binding, in the boar proteins dimerization precludes this activity. Remarkably, the major bovine seminal plasma heparin-binding protein, BSP-A1/2, which is structurally related to stallion HSP-1 and HSP-2 [13,14], was also eluted as aggregated molecules (M_r 60 000–120 000) during gel filtration [28].

The effect of glycosylation on the high-order structure of glycoproteins is not without precedent. The three-dimensional structure of the lactose complex of the legume *Erythrina corallodendron* lectin (EcorL), a dimer of M_r 30 000 N-glycosylated subunits, has been determined crystallographically [29]. If the tertiary structure of the subunit were similar to that of other legume lectins, but interfered by the bulky N-linked heptasaccharide linked to asparagine-17, which is part of the canonical monomer–monomer interface of the legume lectin family, then the EcorL dimer was forced into a drastically different quaternary structure.

We are currently investigating whether the effect of glycosylation on the assembly of stallion and boar seminal plasma proteins is related to protein–carbohydrate binding or to the modulation of protein–protein interaction, as well as whether similar heparin-binding regulatory mechanism has evolved in other mammalian species. In addition, further studies are underway in our laboratories to establish the biological significance (e.g. species-specific modulation of sperm capacitation) of the glycosylation/aggregation state of seminal plasma proteins.

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