The primary structure of BSP-30K, a major lipid-, gelatin-, and heparin-binding glycoprotein of bovine seminal plasma

Juan J. Calvete[a,*], Karlheinz Mann[b], Libia Sanz[a], Manfred Raida[c], Edda Töpfer-Petersen[a]

[a] *Institut für Reproduktionsmedizin, Tierärztliche Hochschule Hannover, Bünteweg 15, 30559 Hannover-Kirchrode, Germany*
[b] *Max-Planck-Institut für Biochemie, Martinsried, Germany*
[c] *Niedersächsisches Institut für Peptid-Forschung GmbH, Hannover, Germany*

Received 8 October 1996; revised version received 30 October 1996

Abstract BSP-30K is a major acidic glycoprotein of bovine seminal plasma. It displays heparin-, gelatin-, and phospholipid-binding activities. BSP-30K binds to spermatozoa upon ejaculation and is thought to play a role in sperm capacitation. We have determined its amino acid sequence, disulfide bonds, and *O*-glycosylation sites. BSP-30K consists of 158 amino acids arranged in a mosaic structure. BSP-30K has a unique 48-residue N-terminal extension which includes three 7–8- amino acid repeats and the six *O*-glycosylated threonine residues. The polypeptide stretch 49–71 is homologous to type 'A' domains found in heparin-binding proteins from other mammalian species. The C-terminal portion of BSP-30K is organized in a tandem of 40–44-residue domains each sharing the consensus pattern of the gelatin-binding fibronectin type II module. The mosaic structure of BSP-30K suggests that this glycoprotein might be a factor contributing to the different sperm-capacitating effects exerted by heparin in different mammalian species.

Key words: Protein BSP-30K; Heparin-binding protein; Bovine seminal plasma; Primary structure; *O*-Glycosylation; Fibronectin type II domain

1. Introduction

Seminal plasma, a complex mixture of secretions originating from the testis, epididymis, and accessory glands, serves as a vehicle for ejaculated spermatozoa, and contains factors that influence the fertilizing ability of sperm. In the bull, exposure of epididymal spermatozoa to seminal plasma enables sperm to be capacitated in vitro by heparin and to respond to agonists of the acrosome reaction [1]. Seminal plasma increases the number of binding sites for heparin on bovine epididymal sperm [2], and the regulatory role of seminal plasma heparin-binding proteins in capacitation of bovine spermatozoa by heparin has been documented [3,4].

Bovine seminal plasma contains four major acidic proteins, designated BSP-A1, BSP-A2, BSP-A3, and BSP-30K [3,5]. These proteins are secretory products of the seminal vesicles [5,6] and bind to spermatozoa upon ejaculation [7–10]. The binding sites of the four BSP proteins on the sperm surface appear to be lipids: BSP-A1, BSP-A2, and BSP-A3 bind specifically to phospholipids which contain the phosphorylcholine (PC) group [11]. BSP-30K, however, displays a much broader binding specificity. It preferentially binds to choline phospholipids but also interacts with phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidic acid, and cardiolipin [11]. The BSP proteins also bind to heparin [3] and, when the purified proteins were coincubated with bovine epididymal sperm, all of them invoked synergistically and in a concentration-dependent manner in vitro sperm capacitation stimulated by heparin, as measured by the onset of acrosome reactions upon exposure to lysophosphatidylcholine (LC) [12]. Bovine epididymal sperm undergo the LC-induced acrosome reaction only in the presence of BSP proteins and the BSP proteins cannot stimulate the acrosome reaction in the absence of heparin [12]. The mechanism by which these bovine seminal plasma proteins modulate sperm capacitation is unknown. It has been proposed that sperm-bound BSP proteins may interact with heparin-like glycosaminoglycans present in the female reproductive tract in particularly high concentration during the estrous cycle and thereby participate in the modifications of the plasma membrane that occur during capacitation [12,13]. Alternatively, or in addition, BSP proteins could act as intermediate molecules in a heparin-induced signal transduction pathway.

The primary structures of BSP-A1, BSP-A2, and BSP-A3 have been reported [14,15]. BSP-A1, also termed PDC-109 [14] and Major Protein [6], has the same polypeptide chain as BSP-A2 but is *O*-glycosylated [10,16]. The structure of BSP-A1/A2 and BSP-A3 is made up of two homologous domains each displaying the consensus sequence of the fibronectin type II module. Here we report the amino acid sequence and posttranslational modifications of the other major BSP protein, BSP-30K.

2. Methods

2.1. Isolation of BSP-30K

Ejaculate from healthy, reproductively active Holstein bulls was a generous gift of RPN (Rinder Produktion Niedersachsen, Verden, Germany). Spermatozoa were separated from seminal plasma by centrifugation at 1500×*g* for 15 min at room temperature. The supernatant was further clarified by centrifugation at 12000×*g* for 15 min. 400 ml seminal plasma was applied to a heparin-Sepharose CL-6B (Pharmacia) column (3×16 cm) equilibrated in 50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 0.025% sodium azide, pH 7.4. The column was washed until the absorbance at 280 nm reached baseline, and BSP-30K was quantitatively recovered in a fraction eluted with column buffer containing 10 mM *o*-phosphorylcholine (Sigma). This eluate was extensively dialyzed against 25 mM Tris-HCl, 1 M NaCl, pH 6.4, and applied to a DEAE-Sephadex A25 column (2.5×20 cm) equilibrated in the same buffer. BSP-30K, recovered in the flow-through eluate, was dialyzed against water, lyophilized, and purified by size-exclusion chromatography on a 16×60 cm HiLoad Superdex 75 (Pharmacia) column equilibrated in 20 mM sodium phosphate, 150 mM NaCl, 0.1% SDS, pH 7.4. Fractions containing apparently pure BSP-30K, as judged by SDS-polyacrylamide gel electrophoresis [17], were extensively dialyzed against MilliQ water and lyophilized.

*Corresponding author. Fax: (49) (511) 953 8504.
E-mail: jcalvete@repro.tiho-hannover.de

This paper is dedicated to the memory of Prof. Karl Heinz Scheit.

PII S0014-5793(96)01310-5

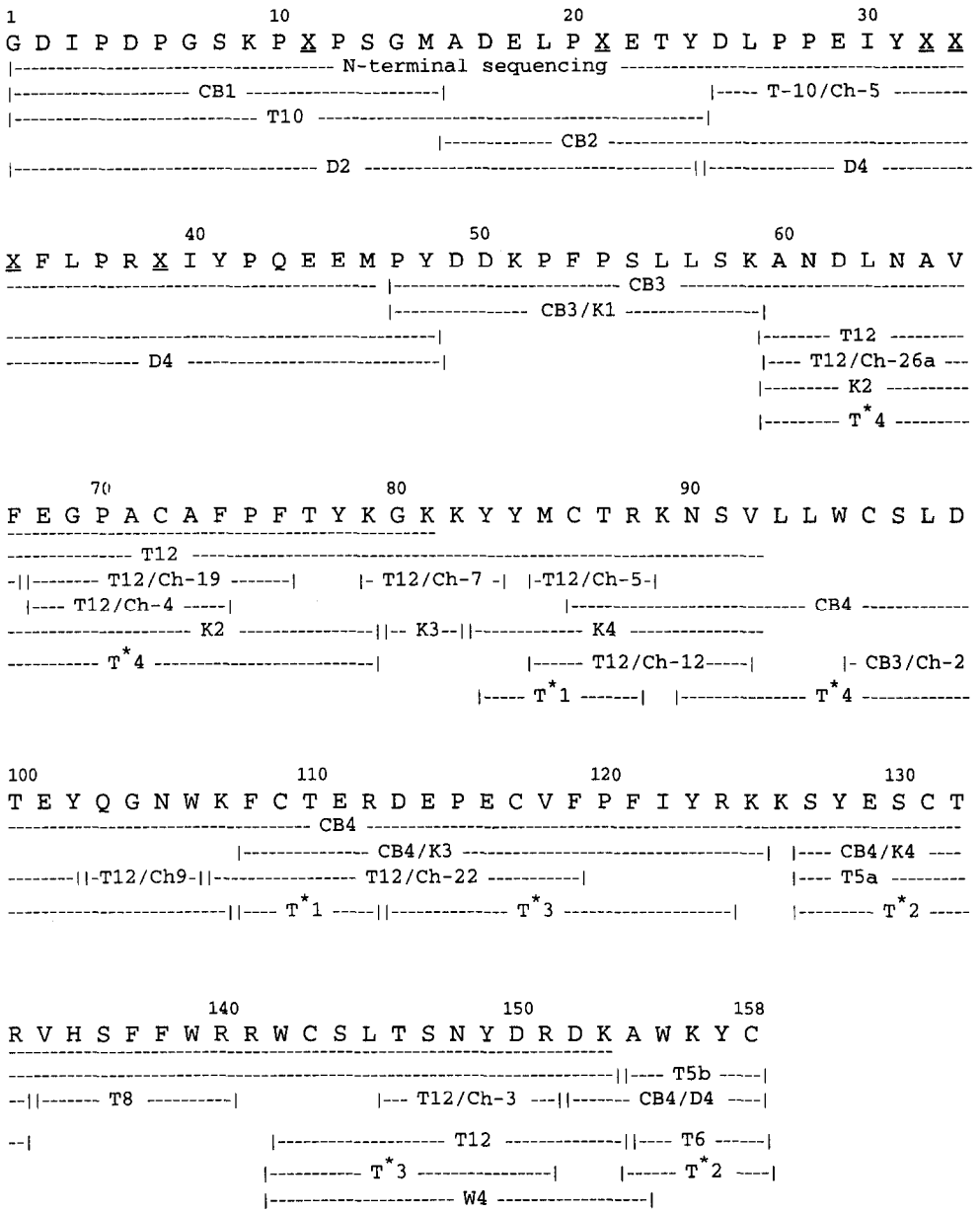

Fig. 1. The amino acid sequence of BSP-30K. CB, T, Ch, D, K, and W are peptides obtained by degradation of pyridylethylated BSP-30K with cyanogen bromide, trypsin, chymotrypsin, endoproteinase Asp-N, endoproteinase Lys-C, and CNBr/DMSO, respectively. T* denotes tryptic peptides of native BSP-30K; X, unidentified residues, which correspond most probably to *O*-glycosylated threonines (see text).

2.2. *Enzymatic and chemical cleavages and isolation of peptides*

Native or reduced and pyridylethylated [18] BSP-30K was dissolved in 50 mM ammonium hydrogen carbonate, pH 8.3, 2 M guanidine hydrochloride to 2–5 mg/ml and was digested at 37°C overnight with trypsin (Sigma) and endoproteinases Lys-C and Asp-N (Boehringer Mannheim) using a 1:100 enzyme:substrate ratio. Peptides were isolated by reversed-phase HPLC on a Super Pac-PepS (Pharmacia) C_{18} column (0.4×25 cm, 5 μm particle size) eluting with a mixture of 0.1% TFA in water (solution A) and acetonitrile (solution B), first isocratically (10%B) for 5 min, followed by 10–45%B for 175 min, and 45–70%B for 10 min. Elution was monitored at 220 nm and peaks were collected manually. Peptides T-10 and T-12 were digested with α-chymotrypsin (1:100 enzyme:substrate ratio) in 0.2 M ammonium hydrogen carbonate, pH 8.3, for 6 h at 30°C. Chymotryptic fragments were separated either by reversed-phase HPLC using a Vydac C_{18} column (0.3×25 cm) eluting at a flow rate of 0.25 ml/min with a gradient of 3–42%B for 160 min, or by size-exclusion chromatography using a Superdex Peptide column HR10/30 (Pharmacia) equilibrated in 0.1% TFA and 25% acetonitrile and run at 0.3 ml/min.

Reduced and pyridylethylated BSP-30K (10 mg/ml in 70% formic acid) was degraded with cyanogen bromide (100 mg/ml final concentration) overnight at room temperature in N_2-flushed tubes in the dark. CNBr fragments were separated by reversed-phase HPLC on a Lichrospher (Merck) RP-100 C_{18} column (0.4×25 cm, 5 μm particle size) eluting at 1 ml/min, isocratically (10%B) for 5 min, followed by 10–50%B for 80 min, and 50–70%B for 20 min. Alternatively, CNBr fragments were separated by size-exclusion chromatography as above. Selected CNBr peptides were subfragmented with endoproteinase Lys-C (Wako Chemicals, Neuss, Germany; 1:50 enzyme:substrate ratio, in 0.1 M ammonium hydrogen carbonate, pH 8.3, containing 4 M urea, at 23°C for 16 h), α-chymotrypsin (Sigma) and endoproteinase Asp-N (as above). Peptides were isolated by reversed-phase HPLC as described above.

Cleavage at tryptophan residues of reduced and pyridylethylated BSP-30K was performed with CNBr in dimethylsulfoxide (CNBr/DMSO) according to [19].

2.3. Determination of thiol groups and disulfide bonds

Titration of free cysteine residues and disulfide bonds was carried out as described [20]. For location of cystine residues, native BSP-30K was degraded with trypsin and the peptides were isolated by reversed-phase HPLC (as above) and characterized by amino acid analysis.

2.4. Analytical methods

Peptides were characterized by N-terminal sequence analysis (using Applied Biosystems sequencers 473A and Procise), amino acid composition (using an Alpha Plus (Pharmacia) analyzer after sample hydrolysis with 6 N HCl for 24 h at 110°C), and carbohydrate content. For amino sugar and neutral sugar analyses the samples were hydrolyzed at 110°C with 4 N HCl for 4 h or 2 N HCl for 2 h, respectively. Sialic acid was determined after sample hydrolysis for 1 h at 80°C with 0.2 N TFA. Monosaccharides were resolved on a CarboPac PA1 column (0.4×25 cm) eluting at 1 ml/min with either 16 mM NaOH (amino and neutral sugars) or 20 mM NaOH in 60 mM sodium acetate and analyzed using a Dionex DX-300 carbohydrate analyzer equipped with a pulsed amperometric detector and the AI-450 chromatographic software [21].

The molecular mass of native BSP-30K was measured by electrospray mass spectrometry using a Sciex API-III LC/MS/MS triple quadrupole instrument.

2.5. Similarity search

Amino acid sequence similarity searches were carried out using the program FASTA [22] 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).

3. Results and discussion

3.1. Isolation of BSP-30K, sequence determination, location of O-glycosylation sites and disulfide bonds

The purification protocol described yielded 20–30 mg BSP-30K from 100 ml of bull seminal plasma. The protein was pure as judged by SDS-polyacrylamide gel electrophoresis, displayed a single peak by reversed-phase HPLC, and showed the N-terminal amino acid sequence GDIPDPGSKPXPSG-MADELPXETYDLPPEIYXXXFLPRXIYPQEEMPY. This sequence does not display discernible similarity with any known protein structure.

The primary structure of BSP-30K was determined from overlapping peptides generated by enzymatic and chemical cleavage of the reduced and pyridylethylated protein (Fig. 1). The residues at positions 11, 21, 32, 33, 34, and 39 could not be identified. However, carbohydrate analysis showed that BSP-30K contains 11 ± 1 mol galactosamine, 9 ± 1 mol galactose, and 11 ± 1 mol sialic acid per mol of protein. Our results are in agreement with the observation of Desnoyers et al. [23] that BSP-30K is the most glycosylated member of the BSP family containing neutral sugars, galactosamine, and sialic acid. Since glucosamine is not present in BSP-30K, we conclude that the six unidentified residues may be *O*-glycosylated. Moreover, amino acid analysis (Table 1) indicated that BSP-30K contains 6 threonine residues more than those calculated from its amino acid sequence (Fig. 1). This strongly suggests that all glycosylated residues of BSP-30K are threonines.

Electrospray mass spectrometric analysis of native BSP-30K yielded broad clusters of protonated quasimolecular ions with 14, 15, and 16 positive charges, from which molecular masses in the range of 25 800–26 400 Da were calculated. This indicates that BSP-30K may consist of a mixture of glycoforms. The isotope-averaged molecular mass of BSP-30K, calculated from the amino acid sequence shown in Fig. 1 and assuming that the six unidentified residues are threonines, is 18 612 Da. Thus, glycosylation may account for 7200–7800 Da, a value which is close to the 6246–7560 Da expected from the carbohydrate content of BSP-30K.

BSP-30K contains 8 cysteine residues (Table 1). No thiol group was detected in native BSP-30K. Digestion with trypsin yielded 4 disulfide-linked peptides (labeled T* in Fig. 1) in a Cys^1-Cys^3, Cys^2-Cys^4, Cys^5-Cys^7, Cys^6-Cys^8 arrangement (Fig. 3).

3.2. Prediction of O-glycosylation sites: the NetOGlyc server

The BSP-30K sequence (assuming all X to be threonine residues) was submitted to the NetOGlyc server [24] for prediction of *O*-glycosylation sites [25]. The only BSP-30K residues which have a high probability of being O-glycosylated (i.e. score >0.5) are Thr^{11} (0.665) and Thr^{39} (0.665). Threonines at positions 21, 23, 32, 33 have scores 0.495, 0.300, 0.350, and 0.174, respectively. With the exception of Ser^{13}, which has a score of 0.305, any other threonine and serine residue have values smaller than 0.17. These results support the claim that the NetOGlyc server may identify correctly 60–95% of the *O*-glycosylated residues and 89–97% of the non-glycosylated residues in two independent test sets of unknown glycoproteins [24]. Although no consensus sequence for *O*-glycosylation has been recognized, *O*-glycosylation sites are often found to cluster and to have a high abundance in the amino-terminal part of the protein [25]. As a whole, these data indicate the presence in bull seminal vesicles of *N*-acetylgalactosyltransferase activity acting at properly exposed BSP-30K threonine residues.

3.3. BSP-30K is a mosaic protein of the fibronectin type II domain-containing protein family

An amino acid sequence similarity search identified BSP-30K as a novel member of the fibronectin type II (Fn-II) domain-containing protein family, which (among others) includes the major heparin-binding proteins of bull and horse seminal plasma (reviewed in [26]). Thus, like bovine PDC-109

Table 1
Amino acid composition of pyridylethylated BSP-30K

Residue	mol%	residues/molecule	Sequence
Asx	10.5	16.0	16
Thr	8.6	13.1	7
Ser	7.1	10.8	11
Glx	8.7	13.2	13
Pro	9.8	14.9	16
Gly	4.4	6.7	6
Ala	4.0	6.1	6
PE-Cys	5.3	8.1	8
Val	2.4	3.6	4
Met	1.7	2.6	3
Ile	2.3	3.5	4
Leu	6.2	9.4	10
Tyr	8.1	12.3	12
Phe	6.6	10.0	10
His	0.4	0.6	1
Lys	8.0	12.2	12
Arg	4.9	7.4	8
Xaa			6
(Trp)			(5)

mol%, mol of residue per 100 mol of total amino acids; residues/molecule, calculated from the amino acid analysis for a 153-residue BSP-30K (158 amino acids minus 5 tryptophan residues); Sequence, residues per molecule calculated from the amino acid sequence shown in Fig. 1. Amino acid residues are in the standard three-letter code. Asx, asparagine+aspartic acid; Glx, glutamine+glutamic acid; PE-Cys, pyridylethylated cysteine; Xaa, unidentified residue.

```
BSP-30K: 1G D I P D P G S K P X P S G M A D E L P X E T Y D L P P E I Y X X X F L P R
                                             I              II              III
BSP-30K:39X I Y P Q E E M P Y D D K P F P S L L S K A N D L N A V F E G P A
HSP-1  : 1D L Q T T G A D H S A T V N P
                  A

BSP-30K:72                                                          C A F P F T Y K G
HSP-1  :16D Q Q L I M - - - - - - - - T K - H S A T V T P E - - - N K C V F P F N Y R G
                          A'
PDC-109: 1D Q D - - - E G V S T E P T Q D G P A - E L P E D E E - - C V F P F V Y R N
BSP-A3 : 1D 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 N K C V F P F I Y G N

                                                                    C (2X) P F X Y (7X)

BSP-30K:81K K Y Y M C T R K N S V L L W C S L D T E Y Q G N W K - F C T E R D E P E C
HSP-1  :43Y R Y Y D C T R T D S F Y R W C S L T G T Y S G I Q V R Y C A A T D Y A K C
PDC-109:33R K H F D C T V H G S L F P W C S L D A D Y V G R W K - Y C A Q R D Y A K C
BSP-A3 :38K K Y F D C T L H G S L F L W C S L D A D Y T G R W K - Y C T K N D Y A K C

                  C (8-10X)          W C  (4X)  D Y (3-5X) W x   Y C              C
                                     + +        + +

BSP-30K:118V F P F I Y R K K S Y E S C T R V H S F F W R R - W C S L T S N Y D R D K A
HSP-1  : 75A F P F V Y R G Q T Y D R C T T D G S L F R I S - W C S V T P N Y D H H G A
PDC-109: 70V F P F I Y G G K K Y E T C T K I G S M W M - S - W C S L S P N Y D K D R A
BSP-A3 : 75V F P F I Y E G K S Y D T C I K I G S T F M - N Y W C S L S S N Y D E D G V

          (2X) P F X Y   (7X)      C      (8-10X)          W C  (4X)  N Y  (3-5X)
                                                           + +        + +

BSP-30K:155W K Y C
HSP-1  :118W K Y C
PDC-109:106W K Y C
BSP-A3 :111W K Y C

          W X Y C
```

Fig. 2. Alignment of the amino acid sequence of BSP-30K with those of equine HSP-1 [28], and bovine PDC-109 [14] and BSP-A3 [15]. The occurrence of unique short repeats (designated I, II, and III) at the N-terminal part of BSP-30K, is indicated. A and A′ are homologous domains found in the equine and bovine proteins but absent in BSP-30K. X denotes an unidentified residue (see Fig. 1); T, *O*-glycosylated threonine. The consensus amino acid residues of the fibronectin type II domain and the spacing between them [37] are shown below the sequence alignment. Residues essential for gelatin binding [27] are labelled with + below the sequence.

and BSP-A3 and equine HSP-1, BSP-30K contains a tandem of Fn-II domains at the C-terminal part of the protein (Figs. 2 and 3). Each of these repeats contains the conserved disulfide bridge pattern of the Fn-II domain, and most of the amino acid residues which have been identified by alanine scanning mutagenesis to be critical for gelatin binding of the fibronectin type II domain of human 92 kDa type IV collagenase [27]. In BSP-30K, the residue at position 99 is Glu instead of the consensus aspartic acid (Fig. 2). In HSP-1, which, like BSP-30K, binds gelatin [5,28], position 99 is threonine (Fig. 2), further indicating that this amino acid position does not need to be absolutely conserved for gelatin-binding activity.

Comparison of the amino acid sequences of BSP-30K with those of PDC-109, BSP-A3, and HSP-1 shows that beside the conserved Fn-II modules, each of these proteins is built by mosaic structures displaying unique N-terminal extensions (Fig. 3). With the exception of BSP-A3 which is not glycosylated [15], the N-terminal extensions of HSP-1 and PDC-109 are, like that of BSP-30K, *O*-glycosylated at threonine residues (Figs. 2 and 3). The single *O*-glycosylation site of PDC-109 is conserved in HSP-1 [28], but the other glycosylation sites of HSP-1 and BSP-30K are located in unique positions. In addition, BSP-30K contains, starting from the N-terminus, a proline-rich region (residues 1–16) and 3 short amino acid sequence repeats (stretches 17–24, 25–31, and 35–41) (Fig. 2), which make BSP-30K unique among mammalian seminal plasma heparin-binding proteins (Fig. 3).

3.4. Occurrence and biological role of proteins immunologically related to bovine BSP-30K

The biological role of the distinct mosaic architecture and glycosylation pattern of related proteins of different mammals is at present unclear. Moreover, the significance of several different BSP proteins having the same effect on sperm capacitation mediated by heparin is not clear inasmuch as this effect appears to be synergistic and not additive [12]. We have previously shown that glycosylation has an indirect effect on the ligand-binding activities of heparin-binding proteins from stallion [28,29] and boar seminal plasma [30] through modulation of their aggregation state. On the other hand, the biological effect of heparin on sperm physiology is not conserved among mammalian species. Thus, heparin plays a major role in in vitro capacitation of bull sperm [3,4], though in the horse this effect appears to be moderate [31], and no heparin is necessary for in vitro capacitation of porcine spermatozoa. Interestingly, the major seminal plasma heparin-binding

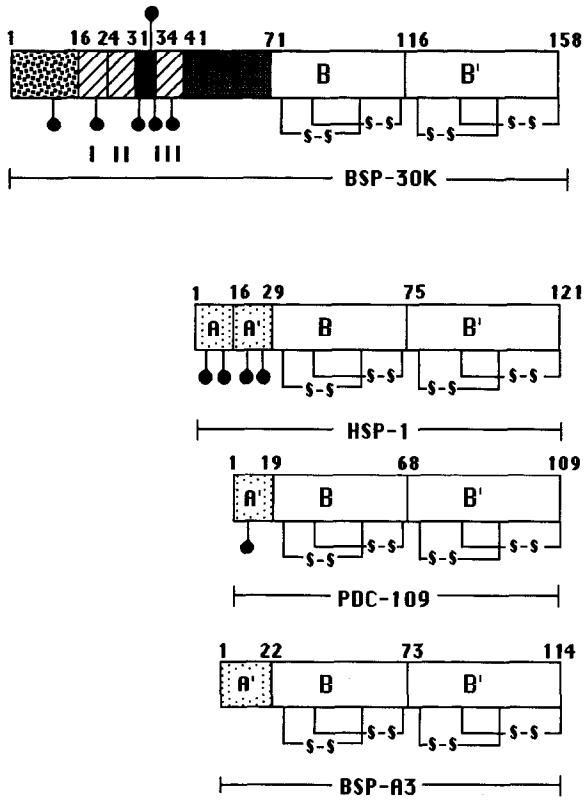

Fig. 3. Scheme of the arrangement of domains in the structures of BSP-30K, HSP-1, PDC-109, and BSP-A3; ●, relative position of *O*-glycosylation sites. S-S, disulfide bridge. I, II, and III represent short amino acid sequence repeats uniquely found in BSP-30K; A, A′ are homologous domains found at the N-terminal part of HSP-1 (both domains) and PDC-109 and BSP-A3 (only domain A′); the polypeptide stretches 1–16 and 41–71 of BSP-30K are sequences without similarity to any known protein.

(Hep+) proteins in these three species belong to only two different protein families: in the bull, BSP proteins account for over 95% of Hep+. In the pig, however, 90% of all Hep+ belong to the spermadhesin protein family [32,33]. The horse represents an intermediate situation containing a large proportion (70%) of HSP-1 and HSP-2 (of the BSP protein family) [26,34] but also a substantial amount of HSP-7 (15%), a member of the spermadhesin protein family [34,35].

Using specific antibodies, Leblond et al. [36] have shown that phosphorylcholine-binding proteins from the seminal fluids of different mammalian species share antigenic determinants with the major proteins of bovine seminal plasma BSP-A1/A2, BSP-A3, and BSP-30K. In the pig, proteins with apparent molecular masses of 14, 64, and 70 kDa were recognized by the anti-BSP-30K antibody. Human seminal plasma showed 58 and 67 kDa BSP-30K-related polypeptides, and 70 kDa proteins were immunodetected in hamster and rat. Thus, proteins immunochemically related to BSP-30K in different mammals may have distinct structural features.

From the above considerations, it seems plausible to propose that the modulatory effect on sperm capacitation exerted by heparin might be due to a combination of the chemical structure and aggregation state of the heparin-binding proteins, and their relative abundance and topography on the sperm surface. The unique modular structure of BSP-30K suggests that this glycoprotein may be one of the factors contributing to the specific bovine spermatozoon phenotype, which in turn may modulate species-specific effects of heparin on sperm capacitation.

Acknowledgements: This work has been financed by Grants 01KY9503 from the Bundesministerium für Forschung, Bildung, und Technologie, Bonn (Germany), Ca209/1-1 from the Deutsche Forschungsgemeinschaft, Bonn (Germany), and PB95-0077 from the Dirección General de Investigación Científica y Técnica, Madrid (Spain).

References

[1] Florman, H.M. and First, N.L. (1988) Dev. Biol. 128, 464–473.
[2] Lee, C.N., Handrow, R.R., Lenz, R.W. and Ax, R.L. (1985) Gamete Res. 12, 345–355.
[3] Chandonnet, L, Roberts, K.D., Chapdelaine, A. and Manjunath, P. (1990) Mol. Repr. Dev. 26, 313–318.
[4] Miller, D.J., Winer, M.A. and Ax, R.L. (1990) Biol. Reprod. 42, 899–915.
[5] Manjunath, P., Sairam, M.R. and Uma, J. (1987) Biosci. Rep. 7, 231–238.
[6] Scheit, K.H., Kemme, M., Aumüller, G., Seitz, J., Hagendorff, G. and Zimmer, M. (1988) Biosci. Rep. 8, 589–608.
[7] Aumüller, G., Vesper, M., Seitz, J., Kemme, M. and Scheit, K.H. (1988) Cell Tissue Res. 252, 377–384.
[8] Nass, S.J., Miller, D.J., Winer, M.A. and Ax, R.L. (1990) Mol. Repr. Dev. 25, 237–246.
[9] Manjunath, P., Chandonnet, L., Leblond, E. and Desnoyers, L. (1994) Biol. Reprod. 50, 27–37.
[10] Calvete, J.J., Raida, M., Sanz, L., Wempe, F., Scheit, K.H., Romero, A. and Töpfer-Petersen, E. (1994) FEBS Lett. 350, 203–206.
[11] Desnoyers, L. and Manjunath, P. (1992) J. Biol. Chem. 267, 10149–10155.
[12] Thérien, I., Bleau, G. and Manjunath, P. (1995) Biol. Reprod. 52, 1372–1379.
[13] Manjunath, P., Soubeyrand, S., Chandonnet, L. and Roberts, K.D. (1994) Biochem. J. 303, 121–128.
[14] Esch, F.S., Ling, N.C., Böhlen, P., Ying, S.Y. and Guillemin, R. (1983) Biochem. Biophys. Res. Commun. 113, 861–867.
[15] Seidah, N.G., Manjunath, P., Rochemont, J., Sairam, M.R. and Chrétien, M. (1987) Biochem. J. 243, 195–203.
[16] Gerwig, G.J., Calvete, J.J., Töpfer-Petersen, E. and Vliegenthart, J.F.G. (1996) FEBS Lett. 387, 99–100.
[17] Laemmli, U.K. (1970) Nature 227, 680–685.
[18] Henschen, A. (1986) in: Advanced Methods in Protein Microsequence Analysis (Wittman-Liebold, B. et al., Eds.), pp. 244–255, Springer, Berlin.
[19] Huang, H.V., Bond, M.W., Hunkapiller, M.W. and Hood, L.E. (1983) Methods Enzymol. 47, 318–324.
[20] Einspanier, R., Krause, I., Calvete, J.J., Töpfer-Petersen, E., Klostermeyer, H. and Karg, H. (1994) FEBS Lett. 344, 61–64.
[21] Anumula, K.R. and Taylor, P.B. (1991) Eur. J. Biochem. 195, 269–280.
[22] Devereux, J., Haberli, P. and Smithies, O. (1984) Nucleic Acids Res. 12, 387–395.
[23] Denoyers, L., Thérien, I. and Manjunath, P. (1994) Mol. Reprod. Dev. 37, 425–435.
[24] NetOGlyc URL address: http://genome.cbs.dtu.dk/netOglyc/cbsnetOglyc.html
[25] Hansen, J.E., Lund, O., Engelbrecht, J., Bohr, H., Nielsen, J.O., Hansen, J.E.S. and Brunak, S. (1995) Biochem. J. 308, 801–813.
[26] Calvete, J.J., Sanz, L., Reinert, M., Dostálová, Z. and Töpfer-Petersen, E. (1995) Mém. Mus. Natl. Hist. Nat. 166, 515–524.
[27] Collier, I.E., Krasnov, P.A., Strongin, A.Y., Birkedal-Hansen, H. and Goldberg, G.I. (1992) J. Biol. Chem. 267, 6776–6781.
[28] Calvete, J.J., Mann, K., Schäfer, W., Sanz, L., Reinert, M., Nessau, S., Raida, M. and Töpfer-Petersen, E. (1995) Biochem. J. 310, 615–622.
[29] Calvete, J.J., Reinert, M., Sanz, L. and Töpfer-Petersen, E. (1995) J. Chromatogr. A 711, 167–173.
[30] Calvete, J.J., Mann, K., Schäfer, W., Raida, M., Sanz, L. and Töpfer-Petersen, E. (1995) FEBS Lett. 365, 179–182.

[31] Varner, D.D., Bowen, J.A. and Johnson, I. (1993) Arch. Androl. 31, 199–207.
[32] Calvete, J.J., Sanz, L., Dostálová, Z. and Töpfer-Petersen, E. (1995) Fertilität 11, 35–40.
[33] Töpfer-Petersen, E., Calvete, J.J., Dostálová, Z., Reinert, M., Waberski, D., Sanz, L. and Hübner, T. (1995) Fertilität 11, 233–241.
[34] Calvete, J.J., Nessau, S., Mann, K., Sanz, L., Sieme, H., Klug, E. and Töpfer-Petersen, E. (1994) Repr. Dom. Anim. 29, 411–426.
[35] Reinert, M., Calvete, J.J., Mann, K., Sanz, L. and Töpfer-Petersen, E. (1996) Eur. J. Biochem. (in press).
[36] Leblond, E., Desnoyers, L. and Manjunath, P. (1993) Mol. Reprod. Dev. 34, 443–449.
[37] Skoestengaard, K., Jensen, M.S., Sahl, P., Petersen, T. and Magnusson, S. (1986) Eur. J. Biochem. 161, 441–453.