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# The N-terminal part of Binder of SPerm 5 (BSP5), which promotes sperm capacitation in bovine species is intrinsically disordered

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ABSTRACT

Bovine BSP5 belongs to the Binder of SPerm (BSP) family. BSP5 plays a role in the bovine sperm capacitation by promoting cholesterol and phospholipid efflux. The variable N-terminal part in the BSP proteins is the uncharacterized region with no known function. Full-length, N-terminal part, and individual fibronectin type II domains of bovine BSP5 were cloned, expressed and purified from *Escherichia coli*. His-S tagged N-terminal part showed large variation in migration on SDS–PAGE in comparison to other constructs. Using mass spectrometry it was demonstrated that the His-S–N-terminal part has the expected molecular mass (13 kDa). The recombinant N-terminal part was sensitive to *E. coli* endogenous proteases during purification. Denaturing purification involving boiling lysis of cells was carried out, as the protein was thermostable. The His-S–N-terminal part lacked structure as determined by CD analysis. Bioinformatics analyses confirmed that the N-terminal part of bovine BSP5 is intrinsically disordered. In addition, bioinformatics analysis indicated that rabbit BSP and multiple forms of BSP proteins of bovine and equine species possess partially or completely disordered N-terminus. The conservation of disorder at the N-terminus in BSP members belonging to different species suggests a role in biological process such as sperm capacitation and/or sperm–egg interactions.



## 1. Introduction

Binders of SPerm (BSP) proteins, considered to be a superfamily, are synthesized exclusively in the mammalian male genital tract [1,2]. Homologues of BSP proteins have been characterized from the seminal plasma of bull, boar, goat, ram, bison, and stallion [3]. BSP homologous DNA sequences have been identified in the genomes of human, mouse, rat, dog, and chimpanzee [1]. BSP-like epididymal glycoprotein has been identified in rabbit [4].

BSP proteins exist in multiple forms (1–6 forms) depending on the species [2]. Bovine BSPs (BSP1, BSP3, and BSP5) are well-characterized proteins in terms of structure and biological functions. Each potentiates *in vitro* high-density lipoproteins (HDL) induced sperm capacitation by promoting cholesterol and phospholipid efflux from the sperm membrane [5]. In addition, the bovine BSP proteins interact with a variety of ligands including heparin, glycosaminoglycans, and apolipoprotein A1 [3]. The bovine BSP proteins also bind to components of semen extenders (egg yolk low-density lipoproteins) used for semen preservation [6]. Other study demonstrated that *in vitro* protein kinase C and tyrosine–protein kinase activities are inhibited by bovine BSP1 [7]. Gwathmey et al. [8] showed that bovine BSP1, BSP3, and BSP5 are involved in the formation of oviduct sperm reservoir thereby prolonging the sperm survival during storage in the oviduct. Nixon et al. [4] reported that antisera raised against the rabbit BSP1 protein blocks the *in vitro* fertilization in a concentration dependant manner indicating the role in sperm–egg interaction. All these studies indicate that BSP proteins are multifunctional.

BSP proteins possess a modular structure made up of non conserved N-terminal extensions, followed by two similar and highly conserved fibronectin type II (Fn2) domains [1,2]. The Fn2 domain is characterized by the presence of several highly conserved aromatic amino acid residues and four invariant cysteines that form intra-domain disulphide bonds. Studies have shown that two Fn2 domains are involved in binding to the sperm membrane but the role of N-terminal part of the BSP proteins has not been investigated. The three-dimensional structure of bovine BSP1 has been solved, but the crystal structure lacks the N-terminal 21 amino acid residues [9]. As a first step toward understanding the functional role of the N-terminal part and ligand binding specificity of individual Fn2 domains we cloned, expressed and purified full-length (FL), N-terminal part, A (first Fn2) domain, B (second Fn2) domain, and AB domains of bovine BSP5. The recombinant N-terminal part showed features of an intrinsically disordered protein, which was supported by the bioin-

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formatics analysis. Furthermore, bioinformatics analyses of BSP superfamily suggested that N-terminal part of BSP protein/s belonging to bovine and rabbit species are intrinsically disordered.

## 2. Materials and methods

### 2.1. Sequence analysis

A total of 10 BSP proteins belonging to six different species [bovine (BSP1, BSP3, and BSP5), porcine (BSP1), equine (BSP1 and BSP2), rabbit (BSP1), mouse (Bsph1 and Bsph2a), and human (BSPH1)] were analyzed. Accession numbers for these proteins are listed in Table 1 of Manjunath et al. [2]. The amino acid composition of the sequence was analyzed with the ProtParam tool (www.expasy.org). The disorder prediction and charge-hydropathy (CH) plot was done using PONDR®. Access to PONDR was provided by Molecular Kinetics (Indianapolis, IN). Other disorder prediction algorithms such as IUPred, RONN, Globplot, and VSL2P were used. Secondary structure content was analyzed using JPRED and Porter. PEST scores were calculated using epestfind algorithm at www.mobyle.pasteur.fr. Rabbit BSP1 sequence was analyzed with NetOGlyc for the prediction of O-linked glycosylation sites (www.cbs.dtu.dk).

### 2.2. Cloning and expression

Bovine seminal vesicle cDNA was used as a template for the polymerase chain reaction (PCR) amplification of FL, N-terminal part, and domains (A, B, and AB) of BSP5. The amplified DNA was purified, treated with BamHI and XhoI and ligated into previously restriction-digested pET32a (Novagen). N-terminal part of bovine BSP5 in addition was cloned into the pET30a vector. DNA sequencing of the inserts revealed perfect identity with the corresponding region of the bovine BSP5 sequence. The pET32a vector encodes for Thioredoxin-His-S.peptide (Trx-His-S) tag whereas pET30a encodes His-S.peptide (His-S) tag. The N-terminal part constructs and the pET32a encoded His-S tagged thioredoxin were expressed in *E. coli* BL21(DE3)pLYS cells, whereas all the other constructs were expressed in *E. coli* Origami B(DE3) cells.

### 2.3. Protein purification

Bacteria were grown in Luria–Bertani medium containing either ampicillin (100 μg/ml) or kanamycin (50 μg/ml). Bacteria were grown at 37 °C. When $OD_{600nm}$ reached ~0.8, induction was carried out at 15 °C with 100 μM isopropyl-β-D-galactopyranoside (IPTG). Harvested cells were suspended in 50 mM Tris–HCl (pH 7.9), 150 mM NaCl, 40 mM imidazole buffer, containing 10% B-PER (v/v), and protease inhibitor cocktail (Roche). In the non-denaturing purification protocol employed for all the constructs, cells were lysed by sonication. In the denaturing purification protocol applied for the N-terminal part, the cell suspension was incubated in boiling water for 20 min, immediately cooled on ice for 5 min, and centrifuged at 15,000 rpm for 60 min at 4 °C. The supernatant was incubated with His-Bind resin (Novagen) at 15 °C for 30 min and then washed with buffers 50 mM Tris–HCl (pH 7.9) containing 0.5 M NaCl, 40 mM imdiazole and 1 M NaCl, and 40 mM imidazole, respectively. The recombinant proteins were eluted off the resin with a buffer containing 50 mM Tris–HCl (pH 7.9), 0.2 M NaCl, and 250 mM imidazole. The fractions containing purified protein were selected using SDS–PAGE analysis, pooled and dialyzed against 50 mM ammonium bicarbonate and lyophilized.

### 2.4. Thermal stability experiments

His-S–N-terminal part and myoglobin (~10 μg each) were kept in boiling water for 30 min and centrifuged at 14,000 rpm for 30 min. The resulting supernatant and pellet were analyzed by 15% SDS–PAGE followed by staining with Coomassie Brilliant Blue.

### 2.5. Mass spectrometry

Determination of mass of the His-S–N-terminal part by matrix-assisted laser desorption/ionization time-of flight (MALDI-TOF) and in-gel tryptic digestion of the protein followed by LC–MS/MS analysis were performed by the proteomics platform service at Genome Quebec, Montreal.

### 2.6. Circular dichroism (CD)

CD measurements were made on a Jasco-810 spectropolarimeter equipped with a temperature control system in a continuous mode. His-S–N-terminal part at 0.25 mg/ml in 10 mM sodium phosphate buffer (pH 7.4) was placed in a cuvette with 0.1 cm path length. Far-UV measurements (an average of five scans) were carried out over wavelength 190–260 nm with 0.5 nm bandwidths at 20 °C. A separate spectrum was generated for the buffer alone and this spectrum was subtracted from spectra taken in the presence of protein.

## 3. Results and discussion

### 3.1. Bioinformatics analyses of the BSP family

The amino acid sequence present before the beginning of the first Fn2 domain was considered as the N-terminal part for the analyses. Inspection of the amino acid sequence of members of the BSP family revealed that the N-terminal part of bovine BSP5, bovine BSP1, and rabbit BSP1 exhibited unusual net charge and pI and was enriched in proline and charged amino acids and de-

**Table 1**
Major characteristics of bovine BSP1, bovine BSP5, rabbit BSP1 and their N- and C-terminal parts.

| Protein | Length | pI[b] | Net charge[b] | Aromatic aa (%) (Phe + Tyr + Trp) | Proline (%) | PEST score[c] |
|---|---|---|---|---|---|---|
| Bovine BSP1 | 109 | 5.08 | −4 | 17.4 | 6.4 | NA |
| Bovine BSP1, N-terminal part | 23 | 3.28 | −10 | 0 | 13.0 | +6.81(residues 1 to 31) |
| Bovine BSP1, C-terminal part | 86 | 8.94 | +6 | 22.1 | 4.7 | ND |
| Bovine BSP5 | 158 | 5.51 | −2 | 17.1 | 10.8 | NA |
| Bovine BSP5, N-terminal part | 71 | 3.91 | −9 | 9.8 | 19.7 | +6.75 (residues 9 to 38) |
| Bovine BSP5, C-terminal part | 87 | 9.06 | +7 | 22.9 | 3.4 | ND |
| Rabbit BSP1 | 455 | 9.09 | +14 | 9.3 | 10.5 | NA |
| Rabbit BSP1, N-terminal part | 367 | 9.47 | +12 | 5.9 | 12.3 | +4.82 (residues 163 to 177) |
| Rabbit BSP1, C-terminal part | 88 | 8.15 | +2 | 22.0 | 3.4 | ND |

aa, amino acids; NA, not applicable; ND, not detected.
[b] The values shown are for the nonglycosylated proteins. These proteins are glycosylated *in vivo* and hence pI and net charge values change after the glycosylation.
[c] The algorithm epestfind assigns a score to each possible sequence found. The PEST score ranges from −55 to +55, with a score above zero denoting a possible PEST region while a value greater than +5 being of particular interest. Only the scores above zero are listed in the table.