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Localization and structural characterization of an oligosaccharide O-linked to bovine PDC-109

Quantitation of the glycoprotein in seminal plasma and on the surface of ejaculated and capacitated spermatozoa

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Abstract

PDC-109 (13 kDa) is the most abundant component, and the major heparin-binding protein, of bovine (*Bos taurus*) seminal plasma. Here, we show that PDC-109 contains a single O-linked oligosaccharide (NeuNAc α(2–6)-Galβ(1–3)-GalNAc-) attached to Thr^{11}. Immunoquantitation of PDC-109 indicates that its concentration in seminal plasma is 15–20 mg/ml. Though PDC-109 is not present on epididymal sperm, ejaculated spermatozoa on average are coated with $(9.5 \pm 0.3) \times 10^6$ molecules of PDC-109/cell. This value remained constant in swim-up sperm and decreased to $(7.7 \pm 0.4) \times 10^6$/spermatozoon after incubation for 24 h in capacitation medium at 39°C. These data substantiate the hypothesis that PDC-109 may be one of the seminal plasma components that enhance the fertilizing capacity of bull spermatozoa upon interaction with heparin-like glycosaminoglycans present in the female genital tract.

Key words: Bovine seminal plasma; PDC-109; Heparin-binding protein; *O*-Glycosylation; Mass spectrometry

1. Introduction

In many mammalian species, sperm capacitation in the female's genital tract, i.e. the complex and poorly understood events which prepare ejaculated spermatozoa to undergo the acrosome reaction in response to interaction with its homologous zona pellucida, is an important control mechanism of the interplay between spermatozoa and the oocyte during fertilization [1]. Seminal plasma, the bulk of the fluid portion of semen, contains the secretions of the male sex accessory glands and appears to play a key role in the development of the sperm capacitated state [2,3].

In bulls, it has been shown that upon short-term exposure of epididymal spermatozoa to seminal plasma, a group of heparin-binding proteins bind to spermatozoa and enhance their fertilizing capacity [4]. The major heparin-binding proteins of bovine seminal plasma are low-molecular-mass proteins termed BSP-A1, BSP-A2, BSP-A3 (15–17 kDa) and BSP-30kDa (28–30 kDa) [5]. They are secreted by the seminal vesicles, bind at ejaculation preferentially to sperm surface phospholipids containing phosphorylcholine, and appear to be immunologically ubiquitous in mammalian species [6–8]. BSP-A1 contain the same polypeptide chain as BSP-A2 but is *O*-glycosylated [9]. Furthermore, BSP-A1/A2 have an amino acid composition indistinguishable from that of bovine 'gonadostatin' PDC-109 [10]. The amino acid sequences of PDC-109 [10], also known as Major Protein (MP) [11], and that of BSP-A3 [12] have been published.

Here, we report the localization and structural characterization of the single O-linked carbohydrate chain of PDC-109 (MP, or BSP-A1/A2). In addition, using a polyclonal antibody, we have determined the concentration of this glycoprotein in bovine seminal plasma, and the number of molecules that bind to the surface of a spermatozoon at ejaculation and those which remain tightly bound during sperm capacitation. Furthermore, heparin-binding was the only ligand-binding ability that could be demonstrated for isolated PDC-109. Altogether, our study may help us to understand the partici-

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pation of PDC-109 in bovine sperm capacitation mediated by glycosaminoglycans.

2. Materials and methods

Epididymal spermatozoa were released from the organs of recently (0.5–1 h) sacrificed mature bulls at the local slaughterhouse. Freshly ejaculated sperm from healthy and reproductive active Holstein bulls was a generous donation of Dr. Pfeilsticker (Rinder Produktion Niedersachsen, Verden, Germany). Spermatozoa were separated from seminal plasma by centrifugation at room temperature at 160 × *g* for 5 min, washed two times with 20 mM phosphate, 135 mM NaCl, pH 7.4 (PBS buffer) and resuspended in the same buffer. The swim-up fraction of ejaculated sperm was obtained as described [13]. In vitro capacitation was done following [14].

PDC-109 was isolated from either seminal plasma or acid extracts of ejaculated sperm [15] by reverse-phase HPLC using a SuperPac Pep-S (Pharmacia) eluting at 1 ml/min with a gradient of 0.1% (v/v) trifluoroacetic acid in (A) water and (B) acetonitrile. Chromatographic conditions were: isocratically (25% B) for 5 min, followed by 25–30% B for 5 min, 30–45% B for 60 min, and 45–70% B for 20 min. PDC-109 was identified by both its amino acid composition (using an Alpha Plus (Pharmacia) amino acid analyzer, after hydrolysis with 6 N HCl for 24 h at 110°C) and N-terminal sequencing (using an Applied Biosystems 473A).

For amino sugars 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 trifluoroacetic acid. Monosaccharides were resolved on a CarboPac PA1 column (4 × 250 mm) eluting at 1 ml/min isocratically with either 16 mM NaOH (amino and neutral sugars) or 20 mM NaOH in 60 mM natrium acetate, and analyzed using a Dionex DX-300 carbohydrate analyzer equipped with a pulsed amperometric detector and the AI-450 chromatographic software [16].

The structure of the oligosaccharide was studied by lectin mapping following [17] using the biotinylated agglutinins (Boehringer Mannheim) of Amaranthus caudatus (ACA), Maackia amurensis (MAA) and Sambucus nigra (SNA), which are specific for the disaccharide Gal β(1–3)-GalNAc, and the α2–3 and α2–6 anomeric forms of the sialic acid-galactose linkage, respectively.

For tryptic digestion, PDC-109 (2–5 mg/ml in 100 mM Tris-HCl, 150 mM NaCl, 1 M guanidine·HCl, pH 8.0) was digested with 1:100 (w/w) TPCK-trypsin (Sigma) overnight at 37°C. Peptides were isolated by reverse-phase HPLC as described above using the following eluting conditions: isocratically (5% B) for 5 min, followed by 5–40% B for 105 min, and 40–60% B for 20 min.

The molecular mass of native PDC-109 was determined by electrospray mass spectrometry using a Sciex API-III LC/MS/MS triple quadrupole instrument.

The ability of purified PDC-109 to bind biotinylated ligands (zona pellucida glycoproteins, heparin, or soybean trypsin inhibitor) was assessed as in [15,18,19].

The amount of PDC-109 in seminal plasma, ejaculated spermatozoa, swim-up sperm, and capacitated sperm, was determined by competitive ELISA. To this end, 50 μl of different dilutions of each sample (in PBS buffer) was mixed with an equal volume of a 1:2,000 (v/v) anti-MP rabbit antibody dilution, and incubated for 1 h at 37°C. The samples were then centrifuged (14,000 × *g*, 15 min) and the free anti-MP antibodies in the clear supernatants were titrated by ELISA. A standard inhibition curve obtained by incubating different amounts of purified MP (PDC-109) with anti-MP was run in parallel in each experiment. For each sample the IC_{50} was defined as the amount of sample (μl fluid, number of spermatozoa, or μg of purified PDC-109) which neutralizes by 50% the binding of the anti-MP antibody solution to 1 μg of immobilized PDC-109. The amount of PDC-109 in a given sample was determined using the following equation:

$$\text{number of PDC-109 molecules} = \left[\frac{IC_{50}\,\text{PDC-109})/(IC_{50}\,\text{Sample})}{M_{\text{PDC-109}}}\right] \times N$$

where $M_{PDC\text{-}109}$ is 1,3444 g/mol and N is Avogadro's number (6.023×10^{23} molecules/mol).

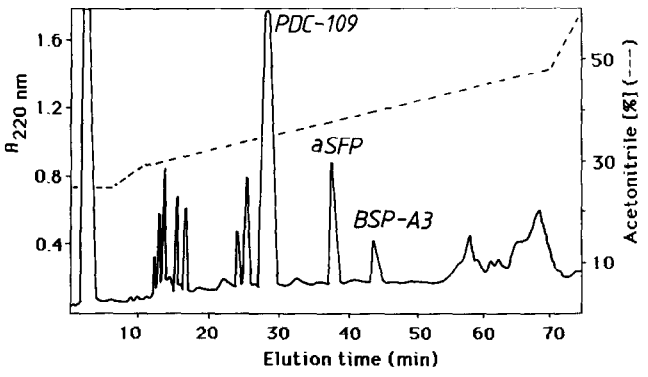

Fig. 1. Isolation of PDC-109 by reverse-phase HPLC. Peaks containing other known proteins are indicated: aSFP, acidic seminal fluid protein [22]; BSP-A3, bovine seminal plasma protein A3 [9,12].

3. Results and discussion

3.1. Location and structural characterization of an O-linked oligosaccharide

Carbohydrate analysis of PDC-109 isolated from either seminal plasma or acid extract of ejaculated sperm (Fig. 1) showed that it contained 1.1 mol galactosamine, 0.9 mol galactose, and 1.0 mol sialic acid per mol protein, suggesting the presence of a single O-linked oligosaccharide per PDC-109 molecule. The same was concluded after analyzing the tryptic peptides of PDC-109 (Fig. 2, Table 1). The peptides covered the complete sequence of PDC-109 but only fragment T5, composed of three disulphide-bonded peptides, contained a carbohydrate structure with the same composition of native PDC-109. It is noteworthy that fragment T6 contained the same structure as T5 without carbohydrate. Therefore, our PDC-109 preparation contained a mixture of glycosylated (major component) and non-glycosylated isoforms. This should explain why Esch et al. [10] did not find any blanck in the PDC-109 sequence while Manjunath and Sairam [9] showed that BSP-A1 (identical to PDC-109) contained 1–2 g neutral sugars, 1–2 g galactosamine, and 1.6–2.5 g sialic acid/100 g glycoprotein.

For localization of the glycosylation site(s), N-terminal amino acid sequence analysis of fragment T5 was performed. The amino acid sequence

```
1          10         20         30
|          |          |          |
DQDEGVSTEPXQDGPAELPEDEE-VFPFVYR
HFD-TVHGSLFPW-SLDADYVGRWK
Y-AQR
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was obtained. The dashes correspond to cysteine residues and the X was a blank at the position where threonine was expected. Taken together, these data indicate that PDC-109 is glycosylated with a single trisaccharide O-linked to threonine 11.

Furthermore, determination of the average molecular

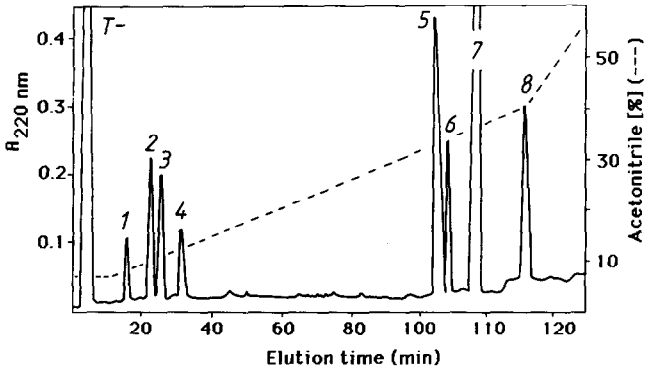

Fig. 2. Reverse-phase isolation of the tryptic fragments of PDC-109. Structural characterization of the isolated peptides is shown in Table 1.

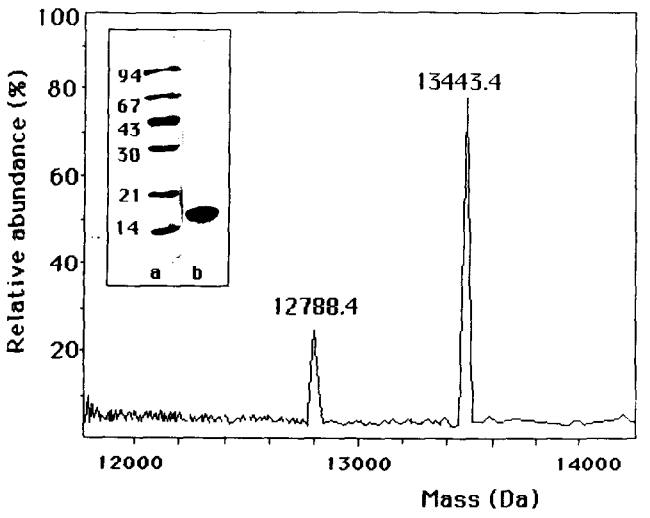

Fig. 3. Deconvoluted mass spectra of HPLC-purified bovine seminal plasma protein PDC-109 (see Fig. 1). The values represent the average of 3 different experiments. Inset, SDS-polyacrylamide (12.5%) gel electrophoresis of purified PDC-109 (lane b). Lane a, molecular mass markers: top to bottom, phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme. Right, their apparent molecular masses (in kDa).

mass of PDC-109 by mass spectrometry showed two species (Fig. 3); the minor one (12,788.4 ± 0.6 Da) corresponds to not glycosylated PDC-109 (calculated 12,788.9 Da). The major species (13,443.4 ± 0.5 Da) is 654.5 Da greater that the mass calculated for the PDC-109 amino acid sequence [10]. The extra mass could correspond, within the experimental error of 0.05%, to a trisaccharide composed of a molecule of each sialic acid, galactose, and *N*-acetylgalactosamine (calculated 656.5 Da).

For structural characterization of the oligosaccharide the binding of biotinylated lectins to immobilized PDC-109 onto ELISA plates was investigated. The glycoprotein bound lectins ACA and SNA, but did not react with MAA. This indicates that the structure of the trisaccharide may be:

Sialic acid α(2–6)-galactose β(1–3)-*N*-acetyl-galactosamine.

Table 1
Characterization of the tryptic peptides of PDC-109 isolated as in Fig. 2, by amino acid and carbohydrate analyses

Fragment	Corresponds to:		GalN	Gal	NANA
			(mol/mol peptide)		
T1	DR	103–104			
	NR	32–33			
T2	AWK	105–107			
T3	YETCTK	80–95			
	YC	108–109			
T4	DYAK	65–68			
T5	DQDEG..	1–31	1.1	0.9	1.0
	HFDCT..	35–57			
	YCAQR	60–64			
T6	DQDEG..	1–31			
	HFDCT..	35–57			
	YCAQR	60–64			
T7	CVFPF..	69–79			
	IGSMW..	86–102			
T8	DQDEG..	1–109	0.9	0.9	1.1

GalN, galactosamine; Gal, galactose; NANA, *N*-acetyl neuraminic acid (sialic acid).

3.2. *Ligand-binding capability and quantitation of PDC-109*

Investigation of the ability of isolated PDC-109 to bind biotinylated ligands (soybean trypsin inhibitor, zona pellucida glycoproteins, and heparin) showed that the glycoprotein had only affinity for heparin (data not shown). Since in the bull, heparin has been shown to bind to the sperm surface and displays a positive effect on sperm capacitation [3–5], we next investigated whether this biological activity could be mediated by PDC-109. We argued that if PDC-109 is involved, it must bind to the sperm surface and remain there during sperm capacitation.

Quantitation of PDC-109 was carried out by competitive ELISA using a rabbit polyclonal antiserum. The concentration of PDC-109 in seminal plasma was 15–20 mg/ml. No PDC-109 could be detected on epididymal sperm. However, an average of $(9.3 \pm 0.3) \times 10^6$ PDC-109 molecules were quantitated on a single ejaculated and washed spermatozoon. This amount remained constant in swim-up spermatozoa and decreased only slightly, $(7.7 \pm 0.4) \times 10^6$ molecules/spermatozoon, upon incubation of swim-up sperm for 24 h in capacitation medium at 39°C. These results indicate that PDC-109 may not be released from the sperm surface during sperm capacitation, and do therefore not support the proposed role for PDC-109 as a decapacitation factor [6].

Our data support the hypothesis that bovine PDC-109 may play a role in the development of the sperm capacitated state induced by heparin-like glycosaminoglycans present in the female's genital tract. The mechanism of this process remains to be elucidated, however. Thus,

Aumüller et al. [20] have reported that Major Protein (PDC-109) binds to the midpiece of spermatozoa to a 65–67 kDa protein duplet and appears to initiate hyperactive sperm motility. On the other hand, Desnoyers and Manjunath [6] have shown that BSP-A1 (PDC-109) binds specifically to phospholipids which contain the phosphorylcholine group, and proposed that this interaction may play a important role in the membrane modification that occurs during capacitation. In addition, the large amount of PDC-109 per spermatozoon (this work), together with the tendency of PDC-109 to polymerize into 5–10-mer aggregates at pH above 7 [9], suggest that PDC-109 may also bind to other PDC-109 molecules bound to the lipid surface and/or to acceptor proteins.

In order to study the molecular architecture of PDC-109 (i.e. the distribution of functional groups involved in its different binding capabilities), we have crystallized the isolated glycoprotein in two different crystal forms (using sodium tartrate and polyethylenglycol 8000 as precipitants). The crystals are currently beeing evaluated.

Structurally, PDC-109 is made up of two homologous domains that are similar to type-II structures present in the gelatin- and heparin-binding domain of fibronectin [12]. Recently, Constantine et al. [21] reported the ^{1}H-NMR solution structure of domain B of PDC-109 (residues 65–109), and noticed 'a depression in the molecular surface structured in part by the exposed edges of partially buried aromatic rings, which may define a ligand-binding site'. Resolution of the three-dimensional structure of the whole PDC-109 may reveal the relative disposition of the A and B domains and may help to identify further putative binding sites within its tertiary structure.

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