

D-fructose-Binding Proteins in Bull Seminal Plasma: Isolation and Characterization

(bull seminal plasma / non-heparin-binding / D-fructose-binding and heparin-binding proteins)

J. LIBERDA¹, M. KRAUS², H. RYŠLAVÁ¹, M. VLASÁKOVÁ¹, V. JONÁKOVÁ², M. TICHÁ¹

¹Department of Biochemistry, Charles University, Prague, Czech Republic

²Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague, Czech Republic

Abstract. The heparin-binding activity of bull seminal plasma proteins was inhibited by D-fructose, D-glucose, inulin and glycogen; D-galactose, dextran and mannan had no effect. While the ejaculated sperm-heparin interaction was not influenced by the presence of saccharides, the heparin-binding activity of epididymal sperm was inhibited by D-fructose. The results of the binding studies were confirmed by affinity chromatography on immobilized heparin followed by elution with monosaccharides. Proteins adsorbed to a heparin-polyacrylamide column and eluted with D-fructose were analyzed by RP HPLC, SDS electrophoresis and by determination of the N-terminal amino-acid sequence. RNAase dimer, PDC-109 and metalloproteinase inhibitor (TIMP-2) were identified.

Mammalian fertilization represents a series of events involving sperm capacitation, gamete recognition and sperm binding to ovum and is mediated by specific interactions between molecules located on the surface of both gametes as well as substances surrounding gametes in the natural environment (Töpfer-Petersen, 1999). Interactions of the lectin type play an important role in some steps of this process (Jonáková et al., 1998; Tichá et al., 1998). Proteins of seminal plasma interact with the sperm surface (Jonáková et al., 1998) and with several substances of different types, including acidic polysaccharides of the heparin type (Liberda et al., 1997a). The binding properties of seminal plasma proteins are

probably influenced by low-molecular-weight components of seminal plasma, e.g. monosaccharides. Seminal plasma of different species contains, besides others, also D-fructose (Tomaszewski et al., 1992). D-fructose present in seminal plasma is not only a source of energy, but it may participate in some steps of the fertilization process. D-fructose was found to inhibit human acrosin (Anderson et al., 1985) or to suppress the acrosome reaction of human spermatozoa (Mori et al., 1993).

The role of individual components participating in the fertilization process is based mainly on their mutual interactions. The function of low-molecular-weight substances, e.g. in seminal plasma, and their influence on the above-mentioned interactions have not been fully explained.

In the present communication, we have studied the effect of D-fructose, present in seminal plasma in relatively high amounts (up to 3 %), on the heparin-binding activity of bull seminal plasma proteins.

Material and Methods

Experimental animals: two experimental bulls, cross-bred (Czech spotted breed x black spotted Holstein), with a satisfactory reproductive function, completely spermatologically examined by 120-min survival test with determination of functional and morphological characteristics, were used to collect semen by artificial vagina. The spermatozoa of the cauda epididymis together with epididymal fluid were obtained from slaughtered bulls.

Sperm preparations: a) ejaculates were centrifuged (300 g, 15 min, room temperature) to separate plasma and sperms. Spermatozoa were washed three times in phosphate-buffered saline (PBS), followed by centrifugation (300 g, 15 min, room temperature). b) Epididymal sperms were prepared by centrifugation of epididymal fluid in the same way as the ejaculated sperms. Sperm suspension was diluted with PBS to the concentration 10^8 cells/ml.

Seminal plasma proteins: ammonium sulphate was added to seminal plasma to reach 80% saturation. Precipitated proteins obtained by centrifugation (3000 g,

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Corresponding author: Věra Jonáková, Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Flemingovo nám. 2, 166 37 Prague 6. Fax: 420 (2) 24310955, 3111274; Tel.: 420 2 20183347; e-mail: vjon@img.cas.cz.

Abbreviations: ABTS – 2,2'-azino-bis(3-methylbenzthiazoline-6-sulphonic acid), ACN – acetonitrile, BSA – bovine serum albumin, ELBA – enzyme-linked binding assay, PBS – phosphate-buffered saline, RP HPLC – reversed-phase high-performance liquid chromatography, SDS – sodium dodecylsulphate, TFA – trifluoroacetic acid.

20 min) were thoroughly dialyzed using Spectra/Por CE Membrane (MWCO = 2000) (Spectrum Medical Industries, Houston, TX) against distilled water and finally against PBS. The obtained protein solution was used for further experiments.

Chemicals: heparin, 2,2'-azino-bis(3-ethylbenzthiazoline-sulfonic acid) (ABTS) and avidin-peroxidase were purchased from Sigma, St. Louis, MO. Immobilon-P-membrane was from Serva, Heidelberg, Germany.

Biotinylated polyacrylamide derivatives of polysaccharides were prepared as described previously (Liberda et al., 1997b). For the preparation of biotinylated protein fractions obtained by affinity chromatography, the procedure described by Maňásková et al. (2000) was used.

Affinity chromatography on heparin-polyacrylamide gel

The PBS solution of bull seminal plasma proteins (corresponding to 600 mg of lyophilized complete seminal plasma) was applied on a heparin polyacrylamide column (3 x 15 cm) according to Tichá et al. (1994), pre-equilibrated with the same buffer. The non-adsorbed proteins (fraction I) were washed with PBS until the absorbance at 280 nm reached the base line. The adsorbed proteins were first eluted with 2% D-fructose solution (fraction II) and then with 3 M NaCl (fraction III). Fractions 5 ml/20 min were collected. Fractions I to III were pooled, desalted on Sephadex G-25 (Pharmacia) in 0.2 % acetic acid and lyophilized.

Reverse-phase high-performance liquid chromatography (RP HPLC)

D-fructose-binding proteins (fraction II) were subjected to inert Biocompatible Quaternary Gradient System of HPLC (Waters, Milford, MA). RP HPLC was performed using a 218 TP 54 Vydac C₁₈ column (4.6 x 250 mm, 5 µm particle size). One mg of the sample in 50 µl of 0.05% trifluoroacetic acid (TFA) was applied and proteins were eluted with a linear gradient of 10–50% acetonitrile (ACN) in 80 min. Protein fractions were lyophilized.

Electrophoresis

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a 15% slab gel according to Laemmli (1970) on MiniProtean II (Bio-Rad). Non-reduced samples of seminal plasma proteins and reduced protein standards were applied. After electrophoresis, the gel was stained with Coomassie brilliant blue R-250.

Blotting and immunodetection

SDS-PAGE was performed and blotting onto a nitrocellulose sheet was carried out according to Towbin et

al. (1979). After blotting, the nitrocellulose sheets were deactivated by 3% bovine serum albumin (BSA) in PBS (pH 7.2) for 1 h at 37°C. The blot was then incubated for 2 h at 37°C with rabbit antibody against human milk lactoferrin (1 : 500 in PBS). After washing in PBS with 0.02% (v/v) Tween 20, the blot was incubated for 1 h at 37°C with peroxidase-labelled swine anti-rabbit antibody in PBS (1 : 3000). The blot was then washed again and developed at 20°C in the dark by the treatment with 0.05% 4-chloro-1-naphthol in 0.01 M Tris-HCl (pH 7.4) containing 0.001 (w/v) CoCl₂ and 0.09% (v/v) H₂O₂. The reaction was stopped after 5 min washing of the blot in distilled water.

N-terminal amino-acid sequence determination

N-terminal amino-acid sequencing was performed using protein sequencer LF 3600 D (Beckmann Instruments) following the Manual Instruction. Proteins isolated by RP HPLC (fractions 1-6) and separated by SDS-PAGE were transferred to the Immobilon-P membrane, visualized by Coomassie blue and subjected to N-terminal amino-acid sequencing. Searches for amino-acid similarities were carried out using the protein sequence deposits in the BLAST-BASIC E-mail Server Databank (Altschul et al., 1990).

Binding studies

Enzyme-linked binding assay (ELBA): microtitre plates were incubated for 1 h at room temperature with 100 µl of BSA solution (1% in PBS, pH 7.2). After extensive washing with washing buffer (PBS), the wells were activated with 100 µl of glutaraldehyde solution (1% in distilled water) for 1 h. After thorough washing with PBS, 100 µl of the PBS solution of seminal plasma proteins (500 µg/ml) or sperm suspension (both ejaculated and epididymal) (10⁸ cells/ml) were applied and incubated for 24 h at 4°C. After extensive washing with distilled water, the wells were deactivated using 100 µl of BSA solution (1% in PBS) for 1 h at room temperature. The solution of biotinylated heparin polyacrylamide derivative or biotinylated protein fractions (100 µg/ml in PBS) was applied to each well (100 µl); the wells were incubated for 2 h at 37°C and then again washed with PBS. Afterwards, 100 µl of avidin-peroxidase solution (0.25 µg/ml) in PBS containing 1% BSA were added to each well and incubated for 1 h at 37°C. After washing, peroxidase was incubated with 250 µl substrate ABTS solution (10 mg/ml in 0.05 M phosphate-citrate buffer, pH 5.0, containing 0.012% sodium perborate). After 30 min of incubation at 37°C, the reaction was stopped by adding 50 µl of 1% SDS. The absorbance was read at 405 nm using a microplate reader (SLT-Spectra, SLT-Labinstruments, Vienna, Austria).

For inhibition studies, the solution of biotinylated heparin polyacrylamide derivative in PBS contained different concentrations of monosaccharides (1.5–100 mM) or polysaccharides (0.02–1 mg/ml).

Results

Inhibition of the heparin-binding activity of bull seminal plasma proteins and sperm by saccharides

The inhibition effect of saccharides on the ability of bull sperm surface and bull seminal plasma proteins to interact with heparin was investigated using ELBA and a biotinylated polyacrylamide derivative of heparin. The heparin-binding activity of bull seminal plasma proteins was inhibited by D-fructose and, in a lesser degree, by D-glucose, inulin and glycogen; D-galactose, dextran and mannan had no effect (Fig. 1).

ELBA assays showed that while the interaction of bull ejaculated sperm with heparin was not influenced by the presence of monosaccharides, the heparin-binding activity of epididymal sperm was inhibited by D-fructose (Fig. 2).

Isolation and characterization of proteins interacting with D-fructose from the heparin-binding fraction of bull seminal plasma

Bull seminal plasma proteins were separated by affinity chromatography on heparin immobilized in polyacrylamide gel into non-heparin-binding proteins (fraction I), D-fructose-binding proteins (fraction II) and remaining heparin-binding proteins (fraction III), and they were characterized by SDS-PAGE (Fig. 3). The results of the ELBA assay with bull seminal plasma proteins were confirmed by affinity chromatography of bull seminal plasma proteins followed by elution with monosaccharides. The amount of proteins eluted with D-fructose was about 8–10 times higher than of those eluted with D-glucose. No proteins were eluted with D-mannose solutions.

Proteins adsorbed to a heparin-polyacrylamide column and eluted with D-fructose (fraction II) were separated by RP HPLC and SDS-PAGE (Fig. 4) and characterized by N-terminal amino-acid sequence determination (Table 1). The D-fructose fraction contained proteins with rel. mol. wt. of 16 000, 17 500, 20 000, 30 000, 48 500, > 60 000. Among proteins of the D-fructose-binding fraction, RNAase dimer, PDC-109 and metalloproteinase inhibitor (TIMP-2) were identified. In the D-fructose-binding fraction (fraction II), N-terminal amino-acid analysis also revealed proteins with the N-terminal sequence not corresponding to known proteins (Table 1). The presence of lactoferrin in fraction II was shown using immunodetection with antibody against human milk lactoferrin. Contrary to RNAase dimer and PDC-109, the metalloproteinase inhibitor and proteins with relative molecular weight of 48 000 to 50 000 were not present in the heparin-binding fraction eluted with 3 M NaCl (fraction III).

Interaction of protein fractions I–III with sperm

The ELBA method was used to study the interaction of the biotinylated protein fractions (fraction I – non-heparin-binding proteins, fraction II – D-fructose-binding proteins, fraction III – the rest of heparin-binding proteins) with ejaculated sperms. The interaction of D-fructose-binding proteins was much weaker than that of the fraction III. The non-heparin-binding fraction of proteins interacted with the sperm surface insignificantly (Fig. 5).

Discussion

Seminal plasma of different species was shown to contain free D-fructose (Oefner et al., 1985; Tomaszewski et al., 1992; Kise et al., 2000). A great attention has been paid to the presence of D-fructose in human seminal plasma: the relationship between seminal D-fructose concentration and sperm characteristics under different physiological and pathological conditions was a subject of many studies (e.g. Tomaszewski et al., 1992; Ludwig et al., 1998; Anrade-Rocha 1999). To the present, a source of energy was considered as the main role for D-fructose in seminal plasma (e.g. Parrish et al., 1989). However, some

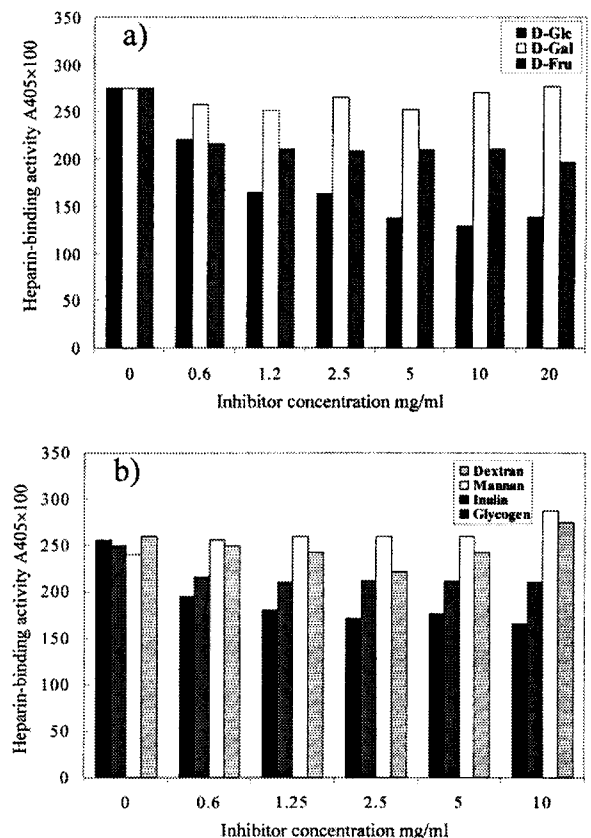


Fig. 1. Inhibition of the heparin-binding activity of bull seminal plasma proteins by monosaccharides (a) and polysaccharides (b). Solutions used: 100 μ g/ml seminal plasma proteins, 0–20 mg/ml monosaccharide (a) or 0–10 mg/ml polysaccharide (b), 50 μ g/ml biotinylated polyacrylamide derivative of heparin. Absorbance at 405 nm – heparin-binding activity.