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Affinity chromatography of bull seminal proteins on mannan–Sepharose

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Abstract

The interaction of bull seminal plasma proteins and sperm with mannan was investigated using an enzyme-linked binding assay (ELBA). A high mannan-binding activity was found in the protein fraction interacting with heparin. Mannan binding to seminal plasma proteins was inhibited by D-mannose and D-fructose, but not by D-mannose-6-phosphate, D-glucose-6-phosphate, ovalbumin and ovomucoid. Mannan inhibited the binding of bovine zona pellucida glycoproteins both to bull sperm and seminal plasma proteins. Yeast mannan immobilized to divinyl sulfone-activated Sepharose was used for the isolation of mannan-binding proteins. The protein components of this fraction were identified on the basis of relative molecular mass determination and N-terminal amino acid sequencing: RNAase dimer, PDC-109 and a protein homologous to BSP-30K (relative molecular mass 14 500). The isolated proteins were characterized by a high zona pellucida binding activity.

Keywords: Proteins; Mannan

1. Introduction

Mammalian fertilization is a series of events that involves a highly coordinated sequence of interactions between molecules located on the surface of both gametes as well as with substances present in the natural environment of the gametes [1]. Interactions of the lectin type play an important role in some steps of this process [2,3].

Saccharide chains of zona pellucida glycoproteins are assumed to bind receptors present on the sperm surface. Although the saccharide ligands were first characterized in mice [4] and pigs [5,6], the ligands of other mammals are not fully recognized. Recently, Amari et al. [7] described the essential role of the non-reducing terminal α-D-mannosyl residues of the N-linked saccharide chain of bovine zona pellucida glycoproteins in sperm-egg binding.

The bovine zona pellucida is composed of three glycoprotein components [8]. They are composed of one kind of neutral saccharide chain (high-mannose-type chain with five mannose residues) and biantennary, triantennary and tetra-antennary acidic complex-type chains containing *N*-acetyllactosamine repeats in non-reducing regions [9]. It has been shown that a high-mannose-type saccharide chain (a major

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neutral chain in bovine zona pellucida) possesses bovine sperm ligand activity [7].

Mannose binding sites of bovine sperm, which might serve to bind to zona pellucida, are exposed or activated at capacitation in the oviduct [10]. Non-capacitated sperm are trapped in the reservoir by binding to L-fucosyl residues in the oviductal epithelium [10,11]. L-Fucose binding sites are lost during capacitation and, at that time, D-mannose binding sites are revealed [10,12]. The bovine seminal plasma protein PDC-109 was identified as the component responsible for formation of the oviduct sperm reservoir [12].

Recent studies have shown that seminal plasma proteins participate in the formation and re-arrangement of the protein coating of the sperm surface, which changes its composition in different phases of the fertilization process [13–15]. As a result of these events, the binding properties of the sperm surface (including saccharide-binding properties) change.

In our previous communication [16] we reported that D-fructose (as a component of seminal plasma) inhibits the heparin-binding activity of bull seminal plasma proteins. The effect of other saccharides was also tested. Yeast mannan, contrary to the other compounds used, activated the heparin-binding activity of bull seminal proteins. The aim of this study was to investigate the saccharide-binding properties of bull seminal plasma proteins. Yeast mannan was immobilized on Sepharose and used for the isolation of proteins participating in the studied interactions.

2. Experimental

2.1. Chemicals

Divinyl sulfone and 1-[3-(dimethylamino)propyl]-3-ethyl-carbodiimide were purchased from Fluka (Buchs, Switzerland). Sepharose 4B was purchased from Pharmacia Biotech (Uppsala Sweden). Heparin, Avidin-Peroxidase, ABTS [1,1-azino-bis(3-ethylbenzthiazoline-sulfonic acid)], D-mannose-6-phosphate, D-glucose-6-phosphate, D-fructose, D-glucose-1-phosphate and ovalbumin were from Sigma (St. Louis, MO, USA). The Immobilon-P-membrane was from SWEVA (Heidelberg, Germany). Ovomucoid was a gift from Dr. K. Bezouška, Department of Biochemistry, Charles University, Praha, Czech Republic.

Saccharomyces cerevisiae cell wall polysaccharide containing mannan and phosphomannan polymers [17] was isolated as described by Haworth [18]. Dephosphorylated mannan was obtained by potato acid phosphatase (EC 3.1.3.2) (Sigma) treatment (at pH 5.0 and 20 °C).

2.2. Bull seminal plasma proteins and sperm

Bull ejaculates were obtained from the Veterinary Research Institute, Brno, Czech Republic. Ejaculates were centrifuged (600 *g*, 20 min, 5 °C) to separate plasma and sperm. Seminal plasma was directly lyophilized, and the sperm was suspended in phosphate-buffered saline (PBS: 20 m*M* phosphate buffer pH 7.4 containing 150 m*M* NaCl) and washed by centrifugation (two times with PBS). The sperm pellet was then lyophilized.

Heparin-binding (H+) and non-heparin-binding (H−) proteins were obtained by affinity chromatography of bull seminal plasma (500 mg lyophilized seminal plasma dissolved in 10 ml PBS) on a Heparin-polyacrylamide column (3×15 cm) [19]. H− proteins were eluted from the affinity column with starting buffer (PBS), adsorbed H+ proteins with 3 *M* NaCl.

2.2.1. Gel chromatography

The solution of lyophilized seminal plasma (25 mg in 2 ml PBS) was applied to a Sephadex G-100 column (2.6×110 cm) equilibrated with the same buffer. The protein peaks, eluted at 20 ml/h, were pooled, desalted on Sephadex G-25 in 0.2% acetic acid and lyophilized.

2.3. Preparation of solubilized zona pellucida glycoproteins

Oocytes were released from bovine ovaries (either fresh or frozen) using a commercial meat grinder with a large amount of ice-cold saline (0.15 *M* NaCl). The homogenate was sieved through nylon screens using the seaving procedure of Hedrick and Wardrip [20]. The oocytes were separated from other particles by centrifugation in a discontinuous Percoll gradient according to Hokke et al. [21]. The oocytes

were collected from 10–20% interface, washed with distilled water and gently homogenized using a small glass homogenizer. Zona pellucida ghosts were then collected on a 40 μm screen and repeatedly washed with PBS. Isolated zona pellucida (ZP) was heat-solubilized in 0.2 *M* $NaHCO_3$, pH 9.0, at 73 °C for 30 min and centrifuged (1500 r.p.m., 10 min, 4 °C). The clear supernatant with a protein concentration of 0.32 mg/ml was used for biotinylation [2].

2.4. Mannan–Sepharose

Immobilized mannan was prepared by the coupling of yeast mannan to divinyl sulfone-activated Sepharose.

2.4.1. Activation of Sepharose 4B with divinyl sulfone [22]

Sepharose 4B (10 ml), washed with distilled water (500 ml), was suspended in 0.2 *M* carbonate buffer pH 10.7 (10 ml) containing divinyl sulfone (1 ml). The suspension was shaken at room temperature for 70 min and then the gel was washed with distilled water.

2.4.2. Coupling yeast mannan

The gel of divinyl sulfone-activated Sepharose (immediately after activation) equilibrated with 0.2 *M* carbonate buffer, pH 10.7, was mixed with a solution of the ligand (1 g in 10 ml of the same buffer) and shaken at room temperature for 20 h. The gels were washed first with distilled water and finally with 0.2 *M* carbonate buffer, pH 10.7. The gels were then suspended in glycine solution (100 mg glycine in 10 ml 0.2 *M* carbonate buffer, pH 10.7), shaken for 2 h at room temperature and again washed with distilled water, 0.05 *M* citrate buffer pH 3.0, and distilled water.

For preparation of the control gel, 2-mercaptoethanol was coupled to divinyl sulfone-activated Sepharose under the same conditions.

2.5. Water-soluble polyacrylamide derivative of mannan

The water-soluble polyacrylamide derivative of yeast mannan was prepared by the coupling of periodate-oxidized mannan to poly(acrylamide–allyl amine) copolymer as described previously [23,24].

Poly(acrylamide–allylamine) copolymer (200 mg), dissolved in distilled water (4 ml), was mixed with a solution of polysaccharides (50 mg in 2 ml distilled water). Then, 0.1 *M* sodium periodate solution (6 ml) was added and the mixture was stirred at laboratory temperature for 2 h. The oxidation reaction was stopped by the addition of ethylene glycol (2 ml). After standing for 15 min at laboratory temperature, sodium cyanoborohydride (80 mg) was added. The obtained solution was dialyzed against distilled water and lyophilized. Copolymer-bound polysaccharide was separated by gel chromatography on a Sephadex G 200 column (1.2×85 cm) in 0.05 *M* NH_4HCO_3. Fractions containing copolymer-bound polysaccharide were collected and lyophilized.

Biotinylation of the polyacrylamide derivative of mannan was carried out as described previously [25].

2.6. Affinity chromatography on mannan–Sepharose

The PBS solution of lyophilized bull seminal plasma (70 mg in 6 ml) was applied on a mannan–Sepharose column (1.5×10 cm) pre-equilibrated with the same buffer. The non-adsorbed proteins were washed with PBS until the absorbance at 280 nm reached baseline. The adsorbed proteins were either eluted directly with 3 *M* NaCl or first eluted with 2% D-mannose solution and then with 3 *M* NaCl. Five milliliter fractions were collected every 20 min. The obtained protein fractions were pooled and dialyzed against distilled water using a Spectra/Por CE Membrane (MWCO 2000) (Spectrum Medical Industries, Houston, TX, USA) and lyophilized.

2.7. SDS electrophoresis

Polyacrylamide gel electrophoresis was performed in the presence of SDS according to Laemmli [26] using a MiniProtean II (Bio-Rad) system and 15% separation gel. 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 onto the Immobilon-P membrane was carried out according to Towbin et al. [27].

2.8. N-terminal amino acid sequence determination

N-terminal amino acid sequencing was performed on a LF 3600 D Protein Sequencer (Beckmann Instruments) following the manufacturer's instructions. Proteins isolated by affinity chromatography on immobilized mannan and separated by SDS–electrophoresis were transferred to the Immobilon-P membrane, visualized by Coomassie Blue and subjected to N-terminal amino acid sequencing. Searches for similarities in amino acids were carried out using the protein sequence deposits in the BLAST-BASIC E-mail Server Databank.

2.9. Binding studies

Microtiter plates were incubated for 1 h at room temperature with 100 μl of bovine serum albumin (BSA) solution (1% in PBS). After extensive washing with washing buffer (PBS), the wells were activated with 100 μl 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 (500 μg/ml) were applied and incubated for 24 h at 4 °C. After extensive washing with distilled water, the wells were deactivated using 100 μl BSA solution (1% in PBS) for 1 h at room temperature. A solution of biotinylated zona pellucida or mannan polyacrylamide derivatives (100 μg/ml) was applied to each well (100 μl). Wells were incubated for 2 h at 37 °C and then again washed with PBS. Then, 100 μl Avidin-Peroxidase solution (0.25 μg/ml) in PBS containing 1% BSA was added to each well and incubated at 37 °C for 1 h. 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 incubation at 37 °C, the reaction was stopped by adding 50 μl of 1% sodium dodecyl sulfate. Absorbance was read at 405 nm using a Microplate reader. Three measurements were performed in parallel.

For inhibition studies, the solution of biotinylated zona pellucida glycoproteins, heparin or mannan polyacrylamide derivatives in PBS contained different concentrations of monosaccharides and their phosphates (1.5–100 m*M*) or polysaccharides and glycoproteins (0.02–1 mg/ml).

3. Results

3.1. Interaction of mannan and bovine zona pellucida glycoproteins with bull seminal plasma proteins and sperm

Biotinylated water-soluble polyacrylamide derivatives of mannan and ELBA (enzyme-linked binding assay) were used to determine the ability of bull seminal plasma proteins and sperm to interact with this ligand. A high mannan-binding activity was found for heparin-binding proteins and sperm, while a very low activity was found for complete seminal plasma and the non-heparin-binding fraction. The dependence of the mannan-binding activity on the concentration of proteins and sperm is shown in Fig. 1. The mannan-binding activity of bull sperm and seminal proteins was inhibited by D-mannose and D-fructose, but not by D-mannose-6-phosphate, D-glucose-6-phosphate, ovalbumin or ovomucoid (Table 1). The interaction of protein with mannan was not dependent on the presence of Ca^{2+}. Acid phosphatase-treated mannan inhibited the binding of the biotinylated native mannan derivative to the H+ fraction of bull seminal plasma proteins. The results indicate that phosphorylated D-mannosyl residues are probably not involved in the interaction of bull seminal plasma proteins with yeast mannan.

In addition to affinity chromatography on immobilized heparin, gel chromatography on Sephadex G-100 in PBS was carried out to separate the bull seminal plasma components. Five fractions with the following relative molecular masses were obtained: >100 000, 65 000 (predominant), 40 000, 26 000 and 17 000 (not shown). Mannan-binding activity was detected in all fractions, however the highest activity was determined for fraction I, followed by fraction II.

A similar approach was used to study the binding of biotinylated bovine zona pellucida glycoproteins to bull seminal plasma proteins and sperm. Labeled zona pellucida glycoproteins interacted both with the sperm surface and the seminal plasma proteins. Heparin-binding proteins exhibited greater zona pel-

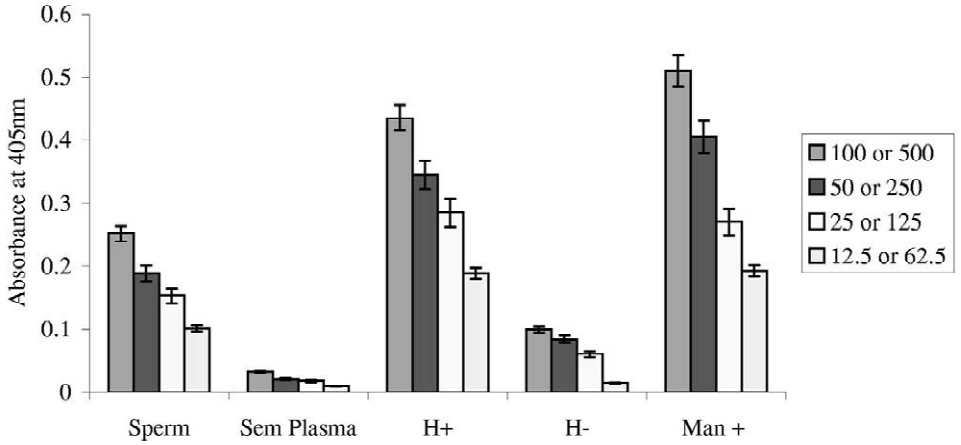

Fig. 1. Mannan-binding activity of bull seminal plasma protein and sperm. The dependence of the binding activity on the concentration of proteins and sperm determined by enzyme-linked binding assay was found to be dose dependent. The absorbance value at 405 nm corresponds to the formation of the product of the peroxidase reaction. Avidin–peroxidase was bound to the protein or sperm complex with the biotinylated derivative of mannan immobilized in the microtiter plate. Abbreviations: H+, heparin-binding proteins; H−, non-heparin-binding proteins; Man+, protein fraction interacting with mannan–Sepharose. Solutions of seminal plasma proteins (12.5–100 μg/ml), the biotinylated polyacrylamide derivative of mannan (100 μg/ml) and sperm suspension (100–500 μg/ml) were used. Binding activity is expressed as absorbance at 405 nm. Vertical bars represent ±standard deviation.

lucida binding activity than the non-heparin-binding fraction. Fig. 2 shows the zona pellucida binding activity as a function of the concentration of proteins and sperm. The highest activity was detected for the mannan- and heparin-binding fraction of proteins. In the presence of yeast mannan, the interaction of zona pellucida glycoproteins both with the sperm and the seminal plasma proteins was inhibited (Fig. 3). The lowest inhibition was observed in the cases of heparin- and mannan-binding proteins. Ovalbumin and ovomucoid did not affect the interaction of zona pellucida with seminal plasma proteins (not shown).

Table 1
Inhibition of the mannan-binding activity of the H+ fraction of bull seminal plasma proteins

Inhibitor	Inhibition
D-Mannose	+++
D-Fructose	++
D-Mannose-6-phosphate	−
D-Glucose-6-phosphate	−
D-Glucose-1-phosphate	(+)
D-Fructose-1-phosphate	−
Ovalbumin	−
Ovomucoid	−

3.2. Isolation and characterization of mannan-binding proteins isolated from bull seminal plasma

Divinyl sulfone-activated Sepharose was used for the preparation of immobilized yeast mannan. Bull seminal plasma was separated on the mannan–Sepharose column into two or three fractions: The M− fraction, which did not interact with the immobilized ligand, and the M+ fraction, which adsorbed to the affinity column and eluted with 3 *M* NaCl (Fig. 4). In an alternative experiment, a proportion of the adsorbed proteins was first eluted with the D-mannose solution and then with 3 *M* NaCl. No proteins were bound to the control gel (2-mercaptoethanol coupled to divinyl sulfone-activated Sepharose).

ELBA tests showed that the mannan-binding fraction of proteins was characterized by a high zona pellucida binding activity (Fig. 2) as well as by a high mannan-binding activity (Fig. 1), compared with the other protein fractions tested (H+, H−, seminal plasma). The interaction of this fraction (M+) with zona pellucida glycoproteins was inhibited by mannan (Fig. 3).

Proteins adsorbed to the mannan–Sepharose col-

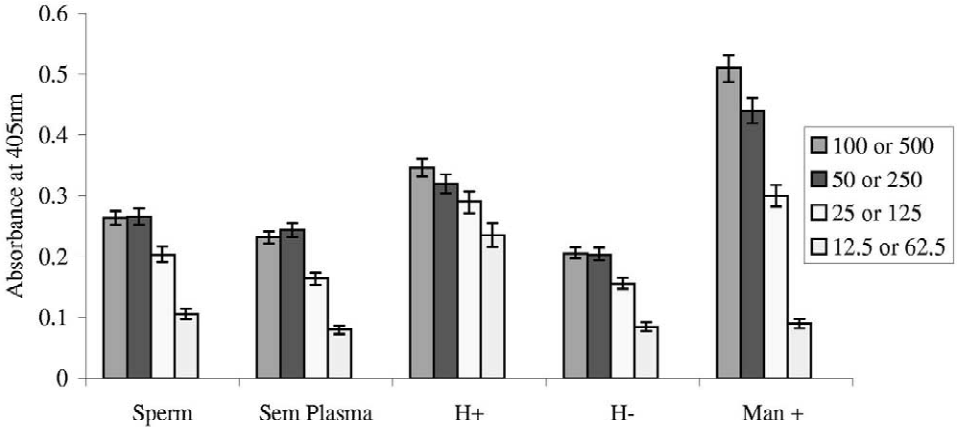

Fig. 2. Binding activity of bovine zona pellucida glycoproteins to bull seminal plasma protein and sperm. The binding activity was found to be dependent on the concentration of proteins and sperm determined by the enzyme-linked binding assay. The absorbance value at 405 nm corresponds to the formation of the product of the peroxidase reaction. Avidin–peroxidase was bound to the protein or sperm complex with the biotinylated derivative of mannan immobilized in the microtiter plate. Abbreviations: H+, heparin-binding proteins; H−, non-heparin-binding proteins; Man+, protein fraction interacting with mannan–Sepharose. Solutions of seminal plasma proteins (12.5–100 μg/ml), biotinylated zona pellucida glycoproteins (100 μg/ml) and sperm suspension (100–500 μg/ml) were used. Binding activity is expressed as absorbance at 405 nm. Vertical bars represent ±standard deviation.

umn (M+ fraction) were characterized by SDS electrophoresis (Fig. 4) and, after electrophoretic separation and blotting to a PVDF membrane, by N-terminal amino acid sequencing. The following proteins were identified in the mannan-binding fraction eluted from the affinity column with 3 *M* NaCl: RNAase dimer, PDC-109 protein and a protein homologous to BSP-30K (Table 2). The protein fraction eluted from the affinity column first with D-mannose solution contained the PDC-109 protein,

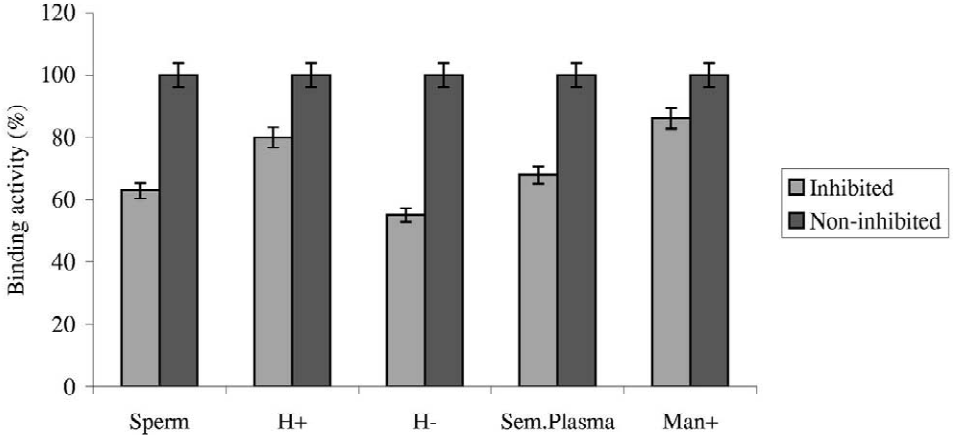

Fig. 3. Inhibition of the bovine zona pellucida binding activity of bull seminal plasma proteins and sperm by mannan. Zona pellucida binding activity was determined using the enzyme-linked binding assay. Binding activity is expressed as a percentage of the non-inhibited activity. Mannan inhibited the zona pellucida interaction both with the seminal plasma protein fraction and the sperm. Abbreviations: H+, heparin-binding proteins; H−, non-heparin-binding proteins; Man+, protein fraction interacting with mannan–Sepharose. Solutions of seminal plasma proteins (100 μg/ml), biotinylated zona pellucida glycoproteins (100 μg/ml), yeast mannan (1 mg/ml) and sperm suspension (500 μg/ml) were used. Vertical bars represent ±standard deviation.

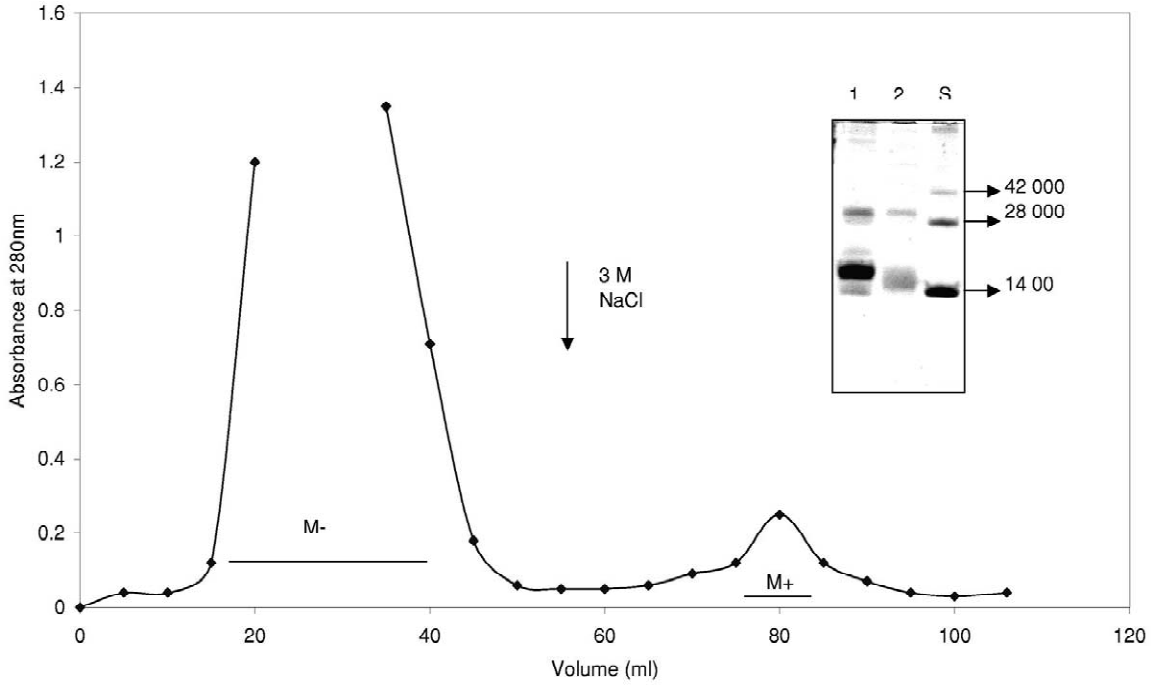

Fig. 4. Affinity chromatography of bull seminal plasma on a mannan–Sepharose column. M−, proteins not interacting with immobilized mannan; M+, proteins adsorbed to mannan–Sepharose. Insert: SDS–polyacrylamide gel electrophoresis of the protein fractions (1, M−; 2, M+) obtained by affinity chromatography under non-reducing conditions. S, standard molecular mass markers: oligomers of lysozyme [28].

the BSP-30K-like protein and only traces of RNAase dimer. The following elution with 3 *M* NaCl yielded mostly RNAase dimer.

4. Discussion

A number of proteins from the seminal plasma of different species have been shown to interact with various types of saccharides (reviewed in Ref. [1]). Heparin or other sulfated polysaccharides and zona pellucida glycoproteins belong to the most studied saccharides. Seminal plasma proteins with affinity for zona pellucida glycoproteins have been identified in a number of mammalian species [1]. Some of them have been characterized biochemically and structurally. However, carbohydrate-binding ability has been demonstrated in only a few instances. It is interesting that, contrary to other species, very little is known about the interaction of isolated bull seminal plasma proteins with bovine zona pellucida glycoproteins. Our results show that zona pellucida

Table 2
Proteins identified in the mannan-binding fraction

Relative molecular mass	N-terminal amino acid sequence	Identified protein
29 000	KESAAAKFER . . .	RNAase dimer
16 000	DQDEGVSTEPTQDGPA . . .	PDC-109
14 000	DQNDLNAVFFGP . . .	Protein homologous to BSP-30K

PDC-109, one of the main acid proteins from bull seminal plasma [29]; BSP-30K, heparin-binding protein from bull seminal plasma [30]; RNAase dimer [31].

binding proteins are mostly present in the heparin-binding protein fraction. The determined interaction of bovine zona pellucida with sperm using the ELBA test is in agreement with the studies of Amari et al. [7].

The ability of isolated protein components to interact with zona pellucida glycoproteins need not necessarily be considered proof of their role as primary gamete adhesive molecules. Under physiological conditions, proteins are present in seminal plasma and probably also in the coating layer on the sperm surface, and in the form of aggregates rather than monomers. The binding properties of these associated forms have been demonstrated, in some cases, to be different from those of isolated monomers [32,33].

Results of ELBA tests as well as of affinity chromatography on mannan–Sepharose showed that bull seminal plasma contains mannan-binding proteins, especially in the fraction interacting with heparin (H+ fraction). The cell wall polysaccharide of *Saccharomyces cerevisiae* contains mannan and phosphomannan polymers [17]. On the basis of our results it can be concluded that phospho-D-mannosyl residues do not participate in the interaction of bull seminal plasma proteins with yeast mannan. The presence of a D-mannose-6-phosphate binding site has been demonstrated in PSP II protein, one of the main proteins of boar seminal plasma. D-Mannose-6-phosphate and heparin-binding sites were found to be distinct, but they possibly overlapped [34]. The mannan-binding activity of bull seminal plasma proteins was also not inhibited by ovomucoid- and ovalbumin-containing oligomannosidic and hybrid-type N-linked carbohydrate chains [35,36].

Mannan inhibition of the zona pellucida binding activity as well as of sperm might indicate the involvement of D-mannose-binding sites in the sperm–egg interaction. These findings are in agreement with the results of Amari et al. [7]. The ability of sperm to interact with mannan, as well as results of its inhibition, is related to the seminal plasma protein layers coating the sperm surface.

In our previous studies [2,25] we used biotinylated polyacrylamide derivatives of acidic polysaccharides (heparin, chondroitin sulfate, dextran sulfate, fucoidan), as well as phosphorylcholine [33], to study the binding properties of seminal plasma proteins. The preparation and application of the labeled mannan derivative has been described in this communication and it was found to be very useful in combination with ELBA in the investigation of the saccharide-binding activities of bull seminal plasma proteins and sperm. The presence of mannan-binding proteins need not be limited only to bull plasma. Proteins with similar saccharide-binding abilities might also be present in the seminal plasma of other species.

Components of the mannan-binding fraction were identified on the basis of relative molecular mass determination and N-terminal amino acid sequencing: RNAase dimer, PDC-109 and a protein homologous to BSP-30 kD. The first two proteins were also found in the protein fraction eluted from immobilized heparin with D-fructose solution [16]. The third protein adsorbed to mannan–Sepharose is a new protein. Its N-terminal amino acid sequence is homologous to that of BSP-30K. The N-terminal amino acid sequence from position 3 is identical to the amino acid sequence of BSP-30K from position 86 [30]. Its relative molecular mass represents about a half of that of BSP-30K. This protein is probably not a product of the proteolytic degradation of BSP-30K protein caused by the action of acrosin. This protease might be found in seminal plasma due to the presence of premature activated sperm, but it is a serine protease, hydrolyzing peptide bonds preferentially after arginine and less after lysine. The cleavage site in the BSP-30K protein (after alanine) would not correspond to the specificity of this enzyme. The new protein, according to its amino acid sequence homology, might belong to the group of BSP proteins, which participate in the fertilization process as capacitation factors, binding to the sperm membrane and potentiating cholesterol and phospholipid efflux from the sperm membrane [37]. This protein might correspond to the protein detected in the non-heparin-binding fraction of bull seminal plasma using immunodetection with anti-BSP.1 antibody [38]. Seminal plasma is a very complex fluid containing a wide variety of both organic and inorganic constituents, with proteins representing an important high-molecular-mass fraction. Detailed characterization of isolated seminal plasma proteins and study of their binding properties will be the first step in understanding their role in the fertilization process.

As in the case of the D-fructose-binding protein fraction obtained from bull seminal plasma [16], the fraction of mannan-binding components contains proteins that were also found in the non-adsorbed fraction. This may be explained by the presence of differently aggregated forms that differ, as already mentioned, in their binding properties. Another explanation might be associated with the different modifications of the protein molecules (e.g. glycosylation, phosphorylation). Differently modified protein forms might exhibit different affinities for the ligand (as suggested for PSP proteins from boar seminal plasma [39]).

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