AGGREGATED FORMS OF BULL SEMINAL PLASMA PROTEINS AND THEIR HEPARIN-BINDING ACTIVITY

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Dedicated to the 50th anniversary of the foundation of the Department of Biochemistry, the first biochemical department in Czechoslovakia.

Heparin-binding activity of bull seminal plasma proteins was shown to be dependent on their aggregation state. The protein fraction interacting with immobilized heparin was characterized by large polydispersity in the region of molecular weight of 60 000–10 000, while that not retained on the affinity carrier was present as aggregates with molecular weight >100 000. Components of heparin-binding and non-heparin-binding fractions were separated by RP HPLC (reversed-phase HPLC) and analyzed by SDS (sodium dodecyl sulfate) electrophoresis and N-terminal sequencing. Size exclusion chromatography of whole seminal plasma and heparin-binding proteins in the presence of D-fructose (as a component of seminal plasma) showed that the region of molecular weights of protein-associated forms was shifted to lower values. An increase of heparin-binding activity of bull proteins, as determined by ELBA (Enzyme-Linked Binding Assay), correlates with a decrease of their aggregation state. The modulation of the aggregation state of bull proteins by seminal plasma components and, in this way, also of their heparin-binding properties suggests possible mechanisms for capacitation mediated by these proteins.

Keywords: Bull seminal plasma proteins; Heparin-binding proteins; Aggregated forms of proteins; Affinity chromatography; Sperm; Fertilization; Seminal plasma.

Mammalian fertilization is a unique event in which morphologically disparate gametes recognize and bind each other and fuse. This event includes highly regulated biochemical interactions: binding of seminal plasma proteins to the sperm surface during ejaculation, interaction of sperm surface proteins with oviductal epithelial cells, sperm capacitation, gamete recognition, primary and secondary binding of the sperm to the ovum, acrosome

reaction of sperm, penetration of the sperm through the zona pellucida of the ovum and fusion of sperm and egg (reviewed by literature[1–5]). Sperm-binding proteins of seminal plasma play roles in these events.

The major proteins found in bull seminal plasma are designated PDC-109 (BSP-A1/-A2), BSP-A3 and BSP-30kDa (collectively called BSP proteins). These proteins are present in seminal plasma in a very high concentration (about 65% of total proteins)[6]. Many studies have been performed to elucidate the biological role of BSP proteins (reviewed by Manjunath and Therien[6]). They bind to the membrane phosphatidylcholine of the sperm surface at ejaculation[7]. Besides, they are bound to high-density lipoprotein (HDL)[8,9] and heparin[10]. HDL and heparin are components of follicular and oviductal fluids and they induce sperm capacitation. Furthermore, studies indicate that BSP proteins potentiate capacitation induced by heparin[11] and by HDL [12]. It was proposed that BSP proteins participate in membrane lipid modification events (cholesterol and phospholipid efflux from the sperm membrane) which occur during capacitation[6].

Another type of studies showed that mannose-binding sites of bovine sperm, which might serve to bind to the zona pellucida, are exposed or activated in capacitation in the oviduct[13]. Non-capacitated spermatozoa are trapped in the reservoir by binding to L-fucosyl residues in the oviductal epithelium[13,14]. L-Fucose-binding sites are lost during capacitation and, at that time, D-mannose-binding sites are revealed[13,15]. Bovine seminal plasma protein PDC-109 was identified as a component responsible for formation of the sperm oviductal reservoir[15].

Even though properties of bull seminal plasma proteins were studied in detail, relatively little is known about aggregated forms of proteins that are present in seminal plasma or on the sperm surface under physiological conditions. The ability of isolated BSP proteins, in particular PDC-109 protein, to form aggregates in the range of molecular weights from 20 000 to 120 000 has already been described by Manjunath and Sairam[16]. The aggregation state of this protein was found to be modulated by phosphorylcholine binding and by solute components[17].

In our previous communication[18], we have shown that D-fructose (as a component of bull seminal plasma) influences the heparin-binding activity of bull seminal plasma proteins and that this saccharide releases a part of proteins adsorbed to immobilized heparin. Changes in the aggregation state of seminal plasma proteins were suggested to be responsible for this phenomenon.

The relationship between heparin binding and the aggregation state of BSP proteins and their biological role is not fully explained. Based on the

results of affinity chromatography on immobilized heparin, Calvete et al.[19] concluded that polydisperse PDC-109, but not its monomeric form displayed the heparin-binding activity.

The subject of the present communication was to study the relationship between the heparin-binding activity and the aggregation state of bull seminal plasma proteins and further factors affecting their aggregation and dissociation.

EXPERIMENTAL

Chemicals

Avidin-peroxidase, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and *N*,*N*-dimethylformamide (DMF) were purchased from Sigma (St. Louis, U.S.A.), D-fructose was a product of Lachema (Brno, Czech Republic). Immobillon-P-membrane and 4-chloro-1-naphthol were products of Serva (Heidelberg, Germany). Nitrocellulose membrane Hybond C-super was a product of Amersham (Vienna, Austria). Sephadex G-75 SF (Superfine) and Sephadex G-25 were products of Pharmacia Fine Chemicals (Uppsala, Sweden). SDS-PAGE standards-broad range and heparin were products of Bio-Rad (Hercules, U.S.A.). Biotinylated polyacrylamide derivative of heparin was prepared as described previously[20].

Bull Seminal Plasma Proteins

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 sperms. Seminal plasma was lyophilized directly.

Affinity Chromatography on a Heparin–Polyacrylamide Column

Bull heparin-binding (H^+) and non-heparin-binding (H^-) proteins were obtained by affinity chromatography of bull seminal plasma on a heparin–polyacrylamide column (2.5 × 4.1 cm)[21]. Thirty milligrams of lyophilized bull seminal plasma were dissolved in 10 ml of 1 mM HCl (pH 3) and 10 ml of phosphate-buffered saline (PBS: 20 mM phosphate (pH 7.4) 150 mM NaCl) and then applied to the column. Proteins non-interacting with heparin (H^- proteins) were eluted from the affinity column with PBS buffer, heparin-binding proteins (H^+ proteins) with 3 M NaCl. In some cases, elution with 2% D-fructose solution in PBS was used prior to elution with 3 M NaCl (D-Fru+ fraction)[18].

The obtained fractions were measured at 280 nm, fractions containing proteins were pooled, desalted on Sephadex G-25 in 0.2% acetic acid and lyophilized.

Size Exclusion Chromatography on Sephadex G-75 SF

Bull seminal plasma (1.6 mg) or protein fractions (1.6 mg) were dissolved in 0.7 ml of 1 mM HCl (pH 3) and 0.2 ml of 0.1 M Tris-HCl containing 0.15 M NaCl (pH 7.2) and applied onto a Sephadex G-75 SF column (1.1 × 98 cm) equilibrated with the same buffer. Fractions eluted at the flow rate 1.4 ml/15 min were measured at 280 nm, the fractions containing proteins were pooled, desalted on Sephadex G-25 in 0.2% acetic acid and lyophilized.

RP HPLC

Protein samples (H^-, H^+) were subjected to the inert Biocompatible Quaternary Gradient system of HPLC (Waters, Milford, U.S.A.). RP HPLC was performed using a 218 TP 104 Vydac C_{18} column (4.6 × 250 mm, 10 μm particle size). One milligram of the sample in 1 ml of 0.05% trifluoroacetic acid (TFA) was applied and proteins were eluted with a linear gradient of 20–50% acetonitrile (ACN) in 60 min. Fractions corresponding to protein peaks were collected and lyophilized.

Electrophoresis and Blotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on 15% slab gel[22]. Non-reduced samples of seminal plasma proteins and reduced protein standards were applied. The molecular weights of the separated proteins were estimated using protein standards run in parallel. Reference molecular markers: phosphorylase B (*M* 97 400) bovine serum albumin (*M* 66 200), egg albumin (*M* 45 000), carbonic anhydrase (*M* 31 000), soybean trypsin inhibitor (*M* 21 500), lysozyme (*M* 14 400), aprotinin (*M* 6500). Electroblotting onto nitrocellulose or Immobilon-P-membrane was carried out according to Towbin et al.[23].

Protein Immunodetection

The nitrocellulose membrane with the transferred proteins was deactivated with 3% BSA in PBS for 2 h. Then, the membrane was washed with 0.02% Tween 20 in PBS and incubated with antibodies against bull seminal plasma inhibitors (BUSI I or BUSI II) (diluted 1:500 with PBS) for 2 h [24]. After washing, the membrane was kept with swine anti-rabbit immunoglobulin coupled to horseradish peroxidase (diluted 1:4000 with PBS) for 1 h. After washing, the membrane was developed in the dark with 0.05% 4-chloro-1-naphthol, 0.001% (w/v) $CoCl_2$ and 0.09% (v/v) hydrogen peroxide in 0.01 M Tris-HCl (pH 7.4). The reaction was stopped after 10 min by washing the membrane in distilled water. All procedures were done at room temperature.

N-Terminal Amino Acid Sequence Determination

N-Terminal amino acid sequencing was performed with Protein Sequencer LF 3600 D (Beckmann Instruments) following the Manual Instruction. Proteins isolated by affinity chromatography on immobilized heparin, separated by RP HPLC and then SDS electrophoresis were transferred onto 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.

Heparin-Binding Studies – Enzyme-Linked Binding Assay (ELBA)

Microtiter plates were incubated at room temperature for 1 h with 100 μl of bovine serum albumin (BSA) solution (1% in PBS). After extensive washing with 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 (12–100 μg/ml) were applied and incubated at 4 °C for 24 h. After extensive washing with distilled water, the wells were deactivated using 100 μl of BSA solution (1% in PBS) at room temperature for

1 h. The solution of biotinylated polyacrylamide derivative of heparin (100 μg/ml) was applied to each well (100 μl); the wells were incubated at 37 °C for 2 h and then again washed with PBS. Afterwards, 100 μl of 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 of substrate ABTS solution (10 mg/ml in 0.05 M phosphate–citrate buffer (pH 5.0) containing 0.012% of sodium perborate). After 30 min of incubation at 37 °C, the reaction was stopped by adding 50 μl of 1% SDS. Absorbance at 405 nm was measured using a micro-plate reader. Three parallel measurements were performed.

RESULTS

Affinity Chromatography of Bull Seminal Plasma Proteins on Immobilized Heparin

Affinity chromatography on heparin–polyacrylamide was used for the separation of bull seminal plasma and two fractions were obtained: protein fraction that was not retained on the affinity column (H^-) and the fraction of heparin-binding proteins (H^+) (Fig. 1a). H^- and H^+ proteins were analyzed by SDS electrophoresis (Fig. 1b). The H^- protein fraction was subjected to repeated affinity chromatography on immobilized heparin under the same conditions; again, two fractions were obtained (Fig. 1c). Experiments with different amounts of bull seminal plasma applied to immobilized heparin showed that this phenomenon was not caused by a low capacity of the affinity carrier.

Protein components of both fractions (H^- and H^+) were further separated by RP HPLC (Fig. 2a) and analyzed by SDS electrophoresis (Fig. 2b), immunodetection using antibodies against bull seminal proteinase inhibitors (BUSI I and BUSI II) and N-terminal amino acid sequencing. The results are summarized in Table I. The main heparin-binding protein of bull seminal plasma was identified both in the heparin-binding and in the non-heparin-binding fractions.

Size Exclusion Chromatography of Bull Seminal Plasma Proteins

Size exclusion chromatography (SEC) on Sephadex G-75 SF was used to study the aggregation state of proteins of bull seminal plasma and its fractions: heparin-binding proteins (H^+) and proteins non-interacting with heparin (H^-) obtained by affinity chromatography (Fig. 1). The course of size exclusion chromatography is presented in Fig. 3. While most protein components of the H^- fraction are present in aggregated form eluted in the void volume of the used column (M > 100 000) (Fig. 3a), the H^+ fraction

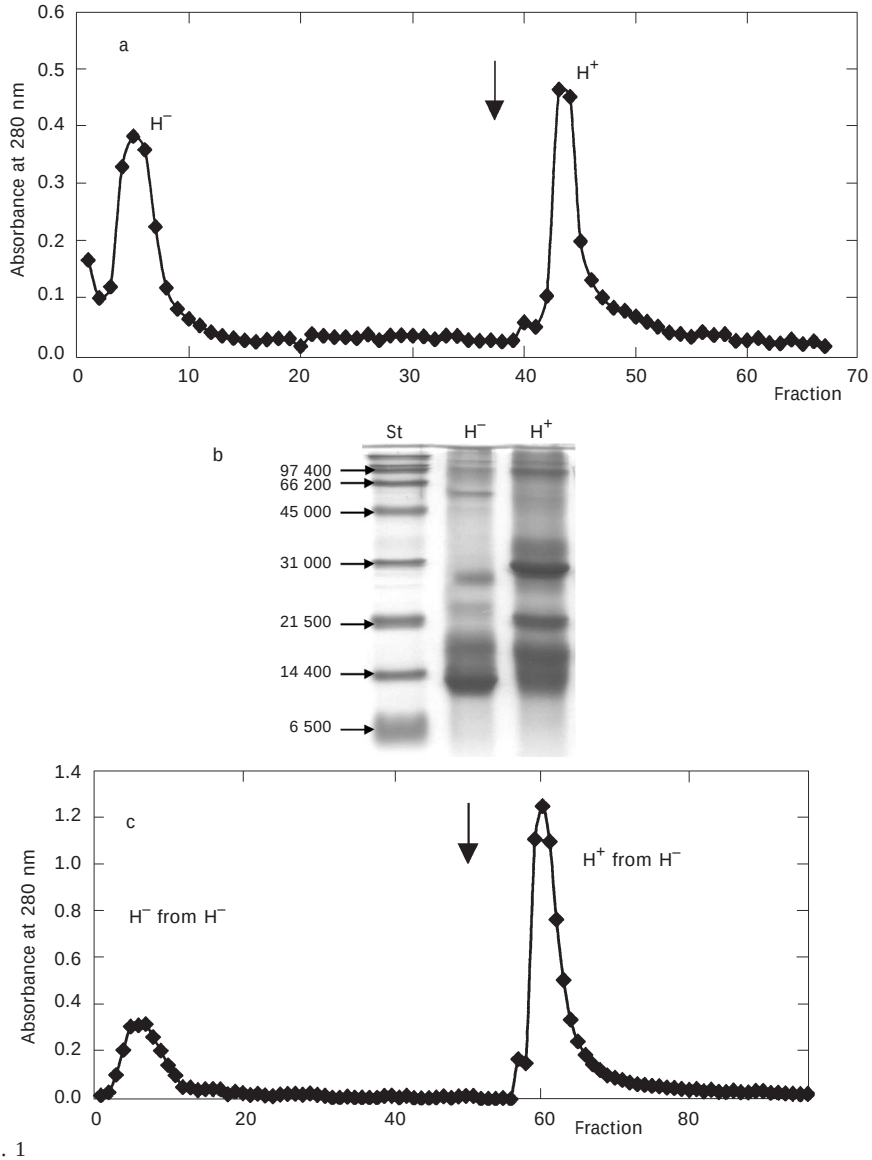

FIG. 1

Affinity chromatography of bull seminal plasma proteins on a heparin–polyacrylamide column. a Bull seminal plasma separated to H^- and H^+ fractions. H^+, heparin-binding proteins; H^-, non-heparin-binding proteins. b SDS electrophoresis of H^+ and H^- fractions. St, standard molecular weight markers: phosphorylase B (*M* 97 400), bovine serum albumin (*M* 66 200), egg albumin (*M* 45 000), carbonic anhydrase (*M* 31 000), soybean trypsin inhibitor (*M* 21 500), lysozyme (*M* 14 400), aprotinin (*M* 6500). c H^- fraction from a – separated H^- and H^+ protein fractions. Arrow, start of the elution with 3 M NaCl

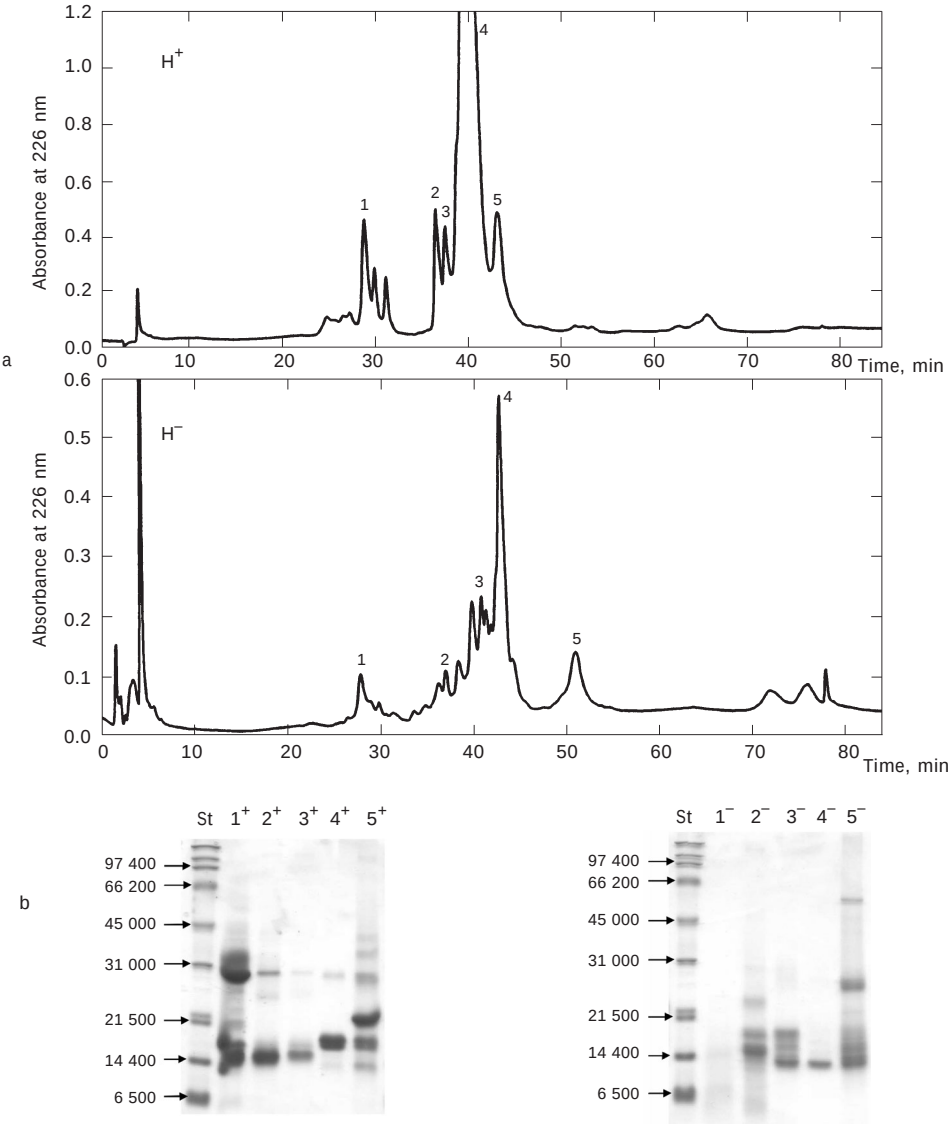

FIG. 2

RP HPLC separation of H^+ and H^- fractions a of bull seminal plasma obtained by affinity chromatography on immobilized heparin. SDS electrophoresis of H^+ and H^- fractions b separated by RP HPLC. St, standard molecular weight markers: phosphorylase B (*M* 97 400), bovine serum albumin (*M* 66 200), egg albumin (*M* 45 000), carbonic anhydrase (*M* 31 000), soybean trypsin inhibitor (*M* 21 500), lysozyme (*M* 14 400), aprotinin (*M* 6500). Fractions 1^+–5^+ were obtained by RP HPLC separation of H^+ proteins, fractions 1^-–5^- by RP HPLC separation of H^- proteins

was polydisperse in respect to molecular weight and only a small part was eluted in the void volume (Fig. 3b). After repeated affinity chromatography of the H^- fraction on immobilized heparin, a part of these proteins were adsorbed to the affinity carrier (Fig. 1c). SEC of this fraction showed that the protein aggregation state was changed in comparison with the original H^- fraction (Fig. 3c).

When SEC separated bull seminal plasma, the region of molecular weights of the main peak was shifted in the direction of lower values of *M* in comparison with the H^+ fraction (Fig. 4). No presence of monomeric forms (*M* of 13 000–16 000) of BSP proteins in the H^+ fraction (Fig. 3) was detected. Only a low amount of proteins was obtained from the elution peak corresponding to *M* of approximately 9000 found in the H^- fraction and the whole seminal plasma.

The effect of D-fructose, a component of bull seminal plasma, on the aggregation of the heparin-binding fraction (H^+) and the fraction that was not adsorbed on immobilized heparin (H^-) is shown in Fig. 5. While the presence of D-fructose had no effect on the behavior of the H^- fraction (Fig. 5a),

TABLE I
Identification of protein components of H^- and H^+ fractions of bull seminal plasma obtained by affinity chromatography on immobilized heparin and RP HPLC

Protein fraction	RP HPLC (Fig. 2)	Molecular weight (SDS electrophoresis)		N-terminal amino acid sequence[a]	Identified protein
H^-	3^-	16 000		DQDEGVSTE....	PDC-109
	2^-	14 000		DQNDLNAV....	Parts of BSP-30-kDa
	2^-	15 000		AVFEGPA....	
	2^-	13 000		N-terminal amino acid blocked	
	4^-	13 000		MDWLPRNT....	aSFP
		8 900	20–29 000	–	BUSI I[b]
		6 200	14–17 000	–	BUSI II[b]
H^+	4^+	16 000		DQDEGVSTE....	PDC-109
	1^+	27 000		KESAAAKF....	RNAase dimer
	3^+	20 000		SXSPVHPQQAFX....	TIMP-2

[a] N-Terminal amino acid sequencing performed after RP HPLC separation, SDS electrophoresis and blotting onto PVDF membrane. [b] Immunochemical detection.
PDC-109 (BSP1/BSP2), main bovine seminal plasma proteins; TIMP-2, metalloproteinase inhibitor; BUSI I and II, bull seminal plasma inhibitors.

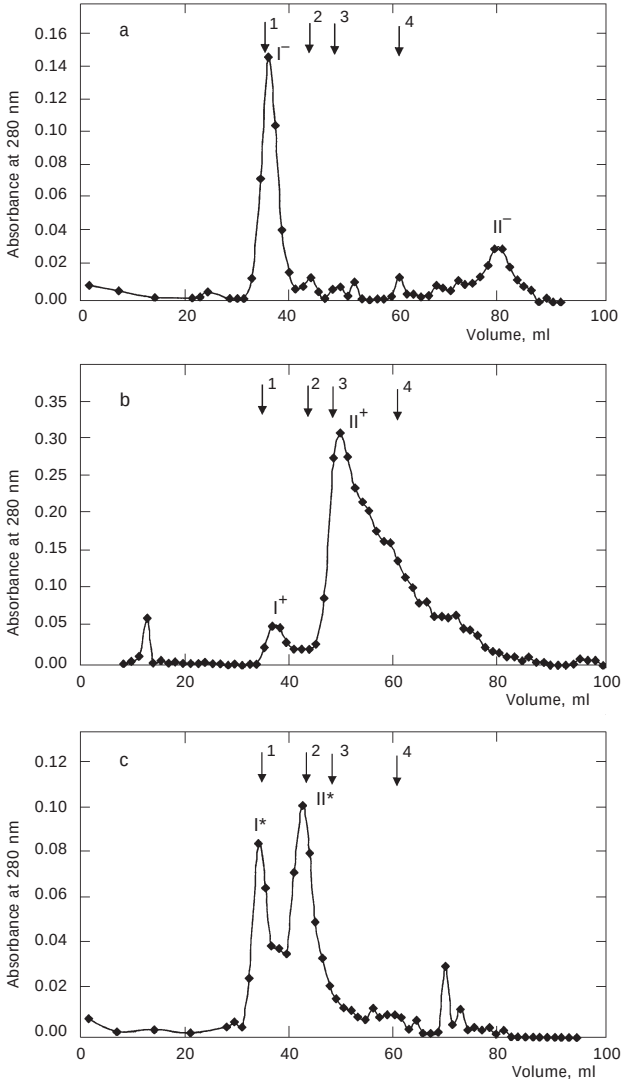

FIG. 3

Size exclusion chromatography on Sephadex G-75 SF of bull seminal plasma proteins and their fractions. Molecular weight markers: 1 Blue Dextran (M > 100 000), 2 bovine serum albumin (M 67 000), 3 egg albumin (M 45 000), 4 chymotrypsinogen (M 25 000). a H^- fraction (Fig. 1a) – separated fractions I^- (M > 100 000) and II^- ($M \approx 9000$). b H^+ fraction (Fig. 1a) – separated fractions I^+ (M > 100 000) and II^+ ($M \approx$ 57–7000). c H^+ fraction obtained after affinity chromatography of H^- fraction on immobilized heparin (Fig. 1b) – separated fractions I* (M > 100 000) and II* ($M \approx$ 66 000). H^+, fraction of heparin-binding proteins; H^-, fraction of non-heparin-binding proteins, obtained by affinity chromatography on immobilized heparin

polydispersity of the H^+ fraction increased in the direction of lower *M*. Disaggregation of the bull H^+ proteins in the presence of D-fructose increased with longer time of the incubation of protein in the saccharide solution (Fig. 5b). The course of SEC showed that H^+ fraction of proteins in the presence of D-fructose contained larger amounts of aggregated forms with lower relative molecular weight similarly to the whole seminal plasma (Fig. 4).

The heparin-binding activity of the obtained fractions was determined using the ELBA assay and biotinylated water-soluble polyacrylamide derivative of heparin. The results are presented in Fig. 6. The heparin-binding activity of the whole seminal plasma and the heparin-binding fraction was significantly higher than that of the fraction not adsorbed to immobilized heparin (H^-). The highest activity was found in the fraction of heparin-binding proteins eluted from immobilized heparin with the D-fructose solution as was described previously[18]. The increase in heparin-binding activity of bull proteins, as determined by ELBA, correlates with the decrease of their aggregation state.

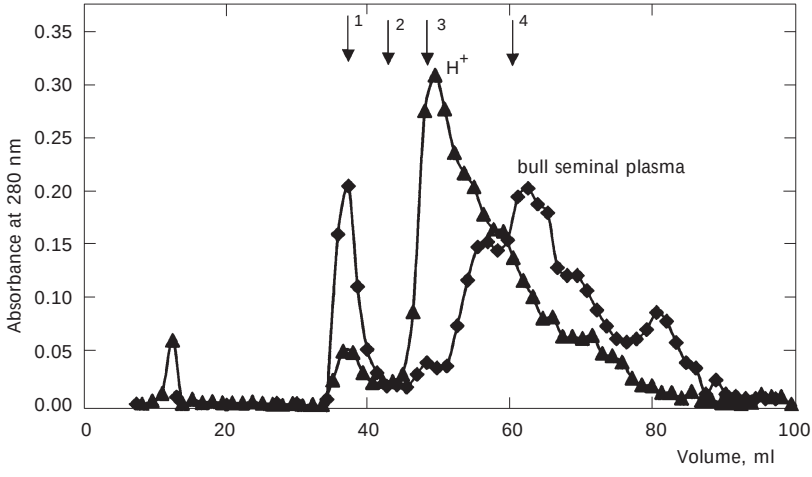

FIG. 4

Size exclusion chromatography of bull seminal plasma and heparin-binding (H^+) proteins on Sephadex G-75 SF. Molecular weight markers: 1 Blue Dextran (*M* >100 000), 2 bovine serum albumin (*M* 67 000), 3 egg albumin (*M* 45 000), 4 chymotrypsinogen (*M* 25 000). Bull seminal plasma, lyophilized seminal plasma; H^+, heparin-binding proteins

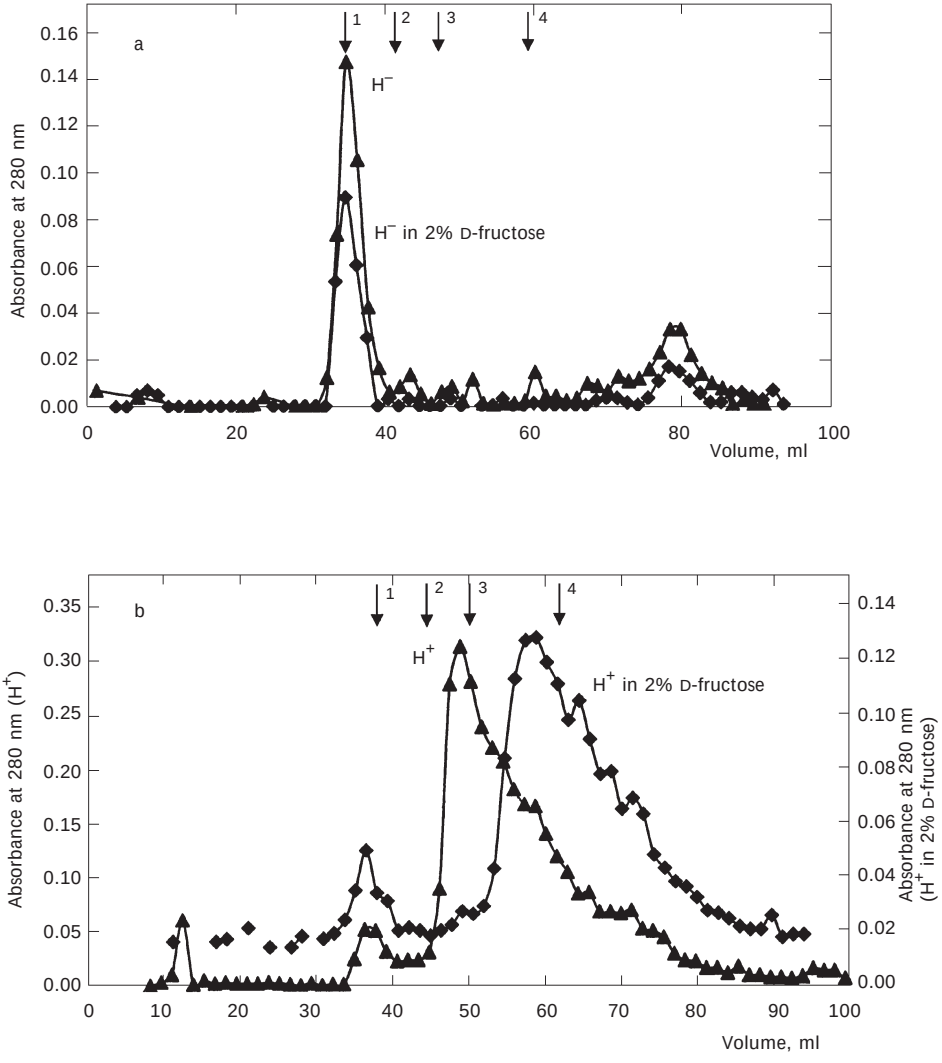

FIG. 5

Size exclusion chromatography of bull seminal plasma proteins in the presence of D-fructose on Sephadex G-75 SF. Molecular weight markers: 1 Blue Dextran (M > 100 000), 2 bovine serum albumin (M 67 000), 3 egg albumin (M 45 000), 4 chymotrypsinogen (M 25 000). a H^- fraction in the presence of 2% D-fructose in eluting buffer. b H^+ fraction in the absence (▲) and in the presence of 2% D-fructose in eluting buffer after 24-h incubation of H^+ proteins in the D-fructose solution prior to chromatography (◆)

DISCUSSION

Affinity chromatography on immobilized heparin was successfully used for the separation of heparin-binding and non-heparin-binding fractions of boar seminal plasma proteins[25,26], which differ in their protein composition. The heparin-binding activity of aggregated forms of proteins from boar seminal plasma corresponds to the activity of isolated monomers[27,28] with only one exception: heterodimer PSP I/PSP II. The heterodimer isolated as non-heparin-binding fraction contains one component (PSP II) with heparin-binding activity. In the case of bull seminal plasma proteins, the interpretation of results of affinity chromatography on immobilized heparin is not simple. This type of affinity chromatography has not usually been used as the first step of the separation of bull seminal plasma proteins[9,16,29].

It is now accepted that BSP proteins participate in the membrane modification events that occcur during sperm capacitation[6,30]. A positive regulatory role of seminal plasma proteins in the sperm capacitation was observed after separation on immobilized heparin only in the case of the heparin-

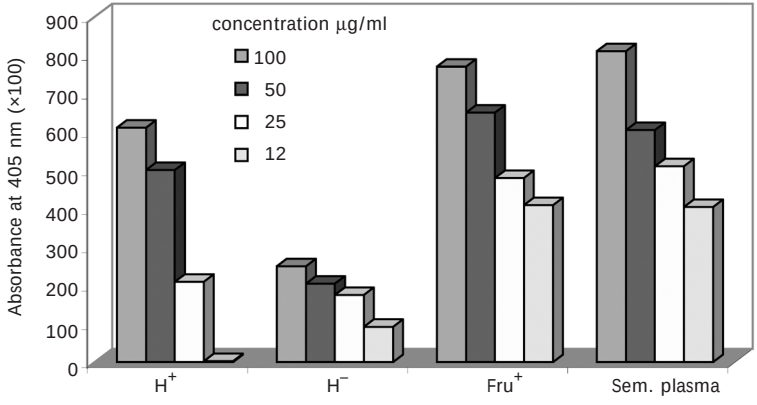

FIG. 6

Heparin-binding activity of bull seminal plasma proteins. The dependence of the binding activity (expressed as absorbance at 405 nm) on the concentration of proteins determined by enzyme-linked binding assay was found to be dose dependent. The value of absorbance at 405 nm corresponds to the formation of the product of peroxidase reaction; avidin-peroxidase was bound to the complex of protein with biotinylated derivative of heparin immobilized in the microtiter plate. H^+, heparin-binding proteins; H^-, non-heparin-binding proteins; Fru+, protein fraction of heparin-binding proteins eluted with D-fructose solution; sem. plasma, seminal plasma. Solutions of seminal plasma proteins (12.5–100 μg/ml), biotinylated polyacrylamide derivative of heparin (100 μg/ml) were used

binding fraction and not of the non-heparin-binding one[30]. Heparin and high-density lipoprotein induce sperm capacitation in the female reproductive tract[31]. BSP proteins, the major products of seminal vesicles, induce initial cholesterol efflux from the sperm membrane and potentiate sperm capacitation after further interaction with either heparin-like glycosaminoglycans or HDL in the oviduct[6].

The tendency of isolated PDC-109 protein to aggregate, as well as the factors that cause dissociation of the formed complexes, e.g. 8 M urea (pH 3.0)[16], or raised concentration of NaCl, $CaCl_2$, EDTA or phosphorylcholine[17] have been described. The above-mentioned substances that increased dissociation of the aggregated forms of proteins were shown to release proteins adsorbed on immobilized heparin[19]. The combination of affinity chromatography and elution using phosphorylcholine solution was used as a method for the isolation of phosphorylcholine-binding proteins from seminal plasma of different species[32–35]. The protein release from immobilized heparin was most probably not caused by competition of these substances for heparin-binding sites, but these substances changed the aggregate state of bull seminal plasma proteins and thus they influenced their heparin-binding activity.

A similar explanation is most probably true in the case of D-fructose elution of heparin-binding proteins of bull seminal plasma adsorbed to immobilized heparin described previously[18]. In the present communication we have shown that the presence of D-fructose influences the aggregation state of heparin-binding proteins, but not of the protein fraction that was not adsorbed to immobilized heparin.

We have used affinity chromatography on a heparin–polyacrylamide column to separate bull seminal plasma proteins into a heparin-binding fraction (H^+) and a non-heparin-binding one (H^-). However, it has been shown that the major protein component was present in both fractions, but that they differed in their aggregation state. It is very difficult to explain the different tendency of the same proteins to form differently aggregated forms. The originally suggested explanation based on the different glycosylation[16] was not confirmed lately. The enzyme removal of saccharide chains did not alter the polydispersity and PDC-109 self-association[17].

The effect of the glycation reaction, due to the presence of a relatively high concentration of D-fructose in bull seminal plasma, cannot be excluded. A difference in the fructosamine content in H^+ and H^- fractions has been observed in a preliminary experiment[36].

Literature data, as well as our results, show that bovine seminal plasma proteins are characterized by a great tendency to form differently aggre-

gated forms. Hydrophobic nature of these proteins[37] participates in this process. Various compounds, including low molecular weight substances present in seminal plasma, influence the aggregation degree of proteins; e.g. above-mentioned D-fructose or compounds of lipid nature. For the isolation of bovine seminal plasma proteins, delipidation of seminal plasma was often used as the first isolation step, e.g. literature[16,37]. In our previous studies[35], we have used two procedures of delipidation (described by Desnoyers and Manjunath[37] or by Liberda et al.[35]). In both cases, the presence of aggregated forms was detected. The ability of different substances present in natural environment of both gametes to change aggregation of BSP proteins may play an important role in the fertilization process.

It can be concluded from the obtained results that: (i) the aggregation state of BSP proteins affects their ability to interact with heparin; (ii) D-fructose, which is a component of bull seminal plasma, causes disaggregation of bull seminal proteins and thus influences their heparin-binding activity.

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