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Isolation of non-heparin-binding and heparin-binding proteins of boar prostate

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Abstract

Proteins of boar prostate secretion were separated by affinity chromatography on heparin–polyacrylamide to non-heparin-binding (H^-) and heparin-binding (H^+) protein fractions. H^- and H^+ fractions were then subjected to RP HPLC. Elution profiles of H^- and H^+ fractions of prostate secretion were compared with those of seminal plasma and the amounts of corresponding proteins were compared. Besides, the isolated proteins were characterized by SDS–PAGE. In the H^- fraction of prostate secretion, PSP I and PSP II spermadhesins and in the H^+ fraction AQN 2 and AWN 1 spermadhesins were found in substantially lower amounts than in seminal plasma. On the contrary, β-microseminoprotein was identified in abundant amounts both in H^- and H^+ fractions of boar prostate secretion. AQN 2 and AWN 1 spermadhesins were proved by their antibodies. Some seminal plasma proteins originating mainly in seminal vesicles could also be secreted by the prostatic gland. β-Microseminoprotein was found to be produced mainly by the prostate.

Keywords: Isolation; Proteins; Heparin

1. Introduction

Seminal plasma is a very complex fluid containing a wide variety of both organic and inorganic constituents; its high-molecular-weight part is represented mostly by proteins. Components of seminal plasma originate in male accessory sexual glands — seminal vesicles, prostatic gland and bulbourethal gland. Protein composition of seminal plasma, as well as protein amounts secreted by individual accessory glands vary from species to species. It is now evident that proteins of seminal plasma affect sperm in its functions and participate in the fertilization process.

Spermadhesins belong to the most studied boar seminal plasma proteins [1–3]. Analysis of amino acid sequences of spermadhesins (AQN, AWN, PSP proteins) showed that they belong to a large family of developmentally regulated proteins [4]. Spermadhesins play diverse roles in the events leading to sperm capacitation, gamete recognition and binding of sperm to the ovum (reviewed by Refs. [5–7]). Many seminal plasma proteins interact with sulphated polysaccharides, such as heparin. The heparin-binding activity of these proteins was also employed in their isolation from boar seminal plasma [2,8].

Prostate is an accessory gland, whose secretion

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contributes to seminal plasma. Generally, little attention has been paid to a systematic study of proteins secreted to seminal plasma by particular sources, except for proteins of human prostate. Acid phosphatase-A2 (PAP) [9,10], prostate-specific antigen (PSA) [11] and PSP-94 (β-microseminoprotein) [12] belong to the well-characterized proteins secreted by human prostate. Much less (except for β-microseminoprotein) is known about prostate proteins from other species. β-Microseminoprotein is a predominant protein of human seminal plasma of the prostate origin [9,12]. It was also found in other body fluids [13].

β-Microseminoprotein isolated from boar seminal plasma has been sequenced [14].

In the present communication we used affinity chromatography on immobilized heparin for the separation of non-heparin-binding and heparin-binding proteins from boar prostate. The composition of these fractions was compared with analogous fractions of boar seminal plasma.

2. Experimental

2.1. Materials

Boar seminal plasma: boar semen was obtained from healthy Large White Pigs from the Insemination Station Nové Mlýny, Czech Republic. Boar seminal plasma was isolated from sperm ejaculate by centrifugation (600 *g*, 20 min, 5°C).

Boar prostatic glands (eight glands) were obtained from slaughtered boars. Prostatic glands were sliced to small pieces and squeezed out. The obtained fluid was diluted with phosphate-buffered saline (PBS; 20 m*M* phosphate buffer pH 7.4, containing 150 m*M* NaCl) to a final volume of 80 ml and centrifuged (3000 *g* for 20 min). The supernatant (designated as ''prostate secretion'') was applied to a heparin–polyacrylamide column.

2.2. Chemicals

Low-molecular-mass protein standards were products of Serva and SDS–PAGE broad-range standards were products of Bio-Rad. Chemicals for RP HPLC were from Merck, swine anti-rabbit immunoglobulin coupled to horseradish peroxidase from Sevac (Prague, Czech Republic), 4-chloro-1-naphthol and Immobillon-P-membrane were purchased from Serva, nitrocellulose membrane Hybond C-super from Amersham. Rabbit antisera against AQN 1 and AWN 1 spermadhesins were prepared in our laboratory as described [2]. The heparin–polyacrylamide column was prepared by immobilization of heparin with water insoluble poly(acrylamide-allylamine) copolymer using the carbodiimide reaction as described by Tichá et al. [15].

2.3. Affinity chromatography on heparin–polyacrylamide

Fifty ml of boar seminal plasma (diluted 1:1 with PBS) were applied to a heparin–polyacrylamide column equilibrated with the same buffer and separated as described by Jonáková et al. [2].

Forty ml of boar prostate secretion diluted by PBS were applied to a heparin–polyacrylamide column (30×75 mm) equilibrated with PBS. Non-adsorbed proteins were washed out with PBS and bound proteins were eluted with 3 *M* NaCl. Fractions containing non-heparin-binding (H^-) and heparin-binding (H^+) proteins were pooled, desalted on Sephadex G-25 in 0.2% acetic acid and lyophilized.

2.4. Reversed-phase high-performance liquid chromatography (RP HPLC)

H^- and H^+ fractions of boar prostate secretion and of boar seminal plasma were subjected to inert Biocompatible Quaternary Gradient system of HPLC (Waters, Milford, USA). RP HPLC was performed using a 218 TP 54 Vydac C_{18} column (4.6×250 mm, 5 μm particle size). The samples of 1 mg in 1 ml of 0.05% trifluoroacetic acid (TFA) were applied and proteins were eluted with a linear gradient of 20–50% acetonitrile (ACN) in 60 min. Protein peaks were collected and lyophilized.

2.5. SDS-electrophoresis and Western blotting

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) was carried out on 15% slab gels. The protein fractions were dissolved in non-reducing buffer and boiled 2.5 min at 100°C

[16]. The molecular masses of the separated proteins were estimated using protein standards run in parallel. Standards were dissolved in reducing buffer (with 2-mercaptoethanol) and boiled 2.5 min at 100°C.

Electroblotting onto nitrocellulose membranes was carried out as described by Towbin et al. [17].

2.6. Protein immunodetection

The nitrocellulose membrane with transferred proteins was deactivated with 3% BSA in PBS for 2 h. The membrane was then incubated with antibody against AQN 1 spermadhesin (dilution of 1:2000 in PBS) or against AWN 1 spermadhesin (dilution of 1:2000 in PBS) for 2 h. After that, the membrane was washed with 0.02% Tween 20 in PBS and kept with swine anti-rabbit immunoglobulin coupled to horseradish peroxidase (dilution of 1:4000 in 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% hydrogen peroxide in 0.01 *M* Tris–HCl, pH 7.4. The reaction was stopped after 10 min by washing the membrane with distilled water. All procedures were done at room temperature. The AQN 1 antibody crossreacts with all spermadhesins of the AQN family [2].

2.7. N-terminal amino acid sequence

The N-terminal amino acid sequence was determined by Edman degradation, and formed phenylthiohydantoin derivates were analysed by HPLC. Protein sequencing was performed automatically using a Protein Sequencer LF3600D (Beckman Instruments).

3. Results and discussion

3.1. Isolation and characterization of proteins from boar prostate secretion

Boar prostate secretion was separated by means of affinity chromatography on heparin–polyacrylamide to H^- and H^+ fractions (Fig. 1). The same conditions were used for the separation of boar seminal plasma. The isolated protein fractions were further subjected to RP HPLC. Compositions of H^- and H^+ fractions of boar prostate secretion and of boar

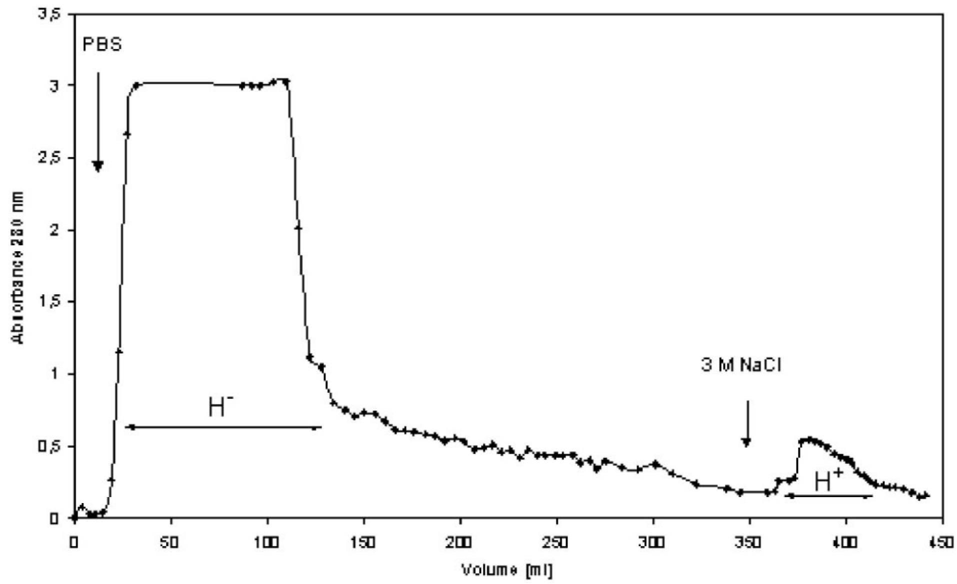

Fig. 1. Affinity chromatography of boar prostate secretion on heparin–polyacrylamide. H^-, non-heparin-binding fraction; H^+, heparin-binding fraction.

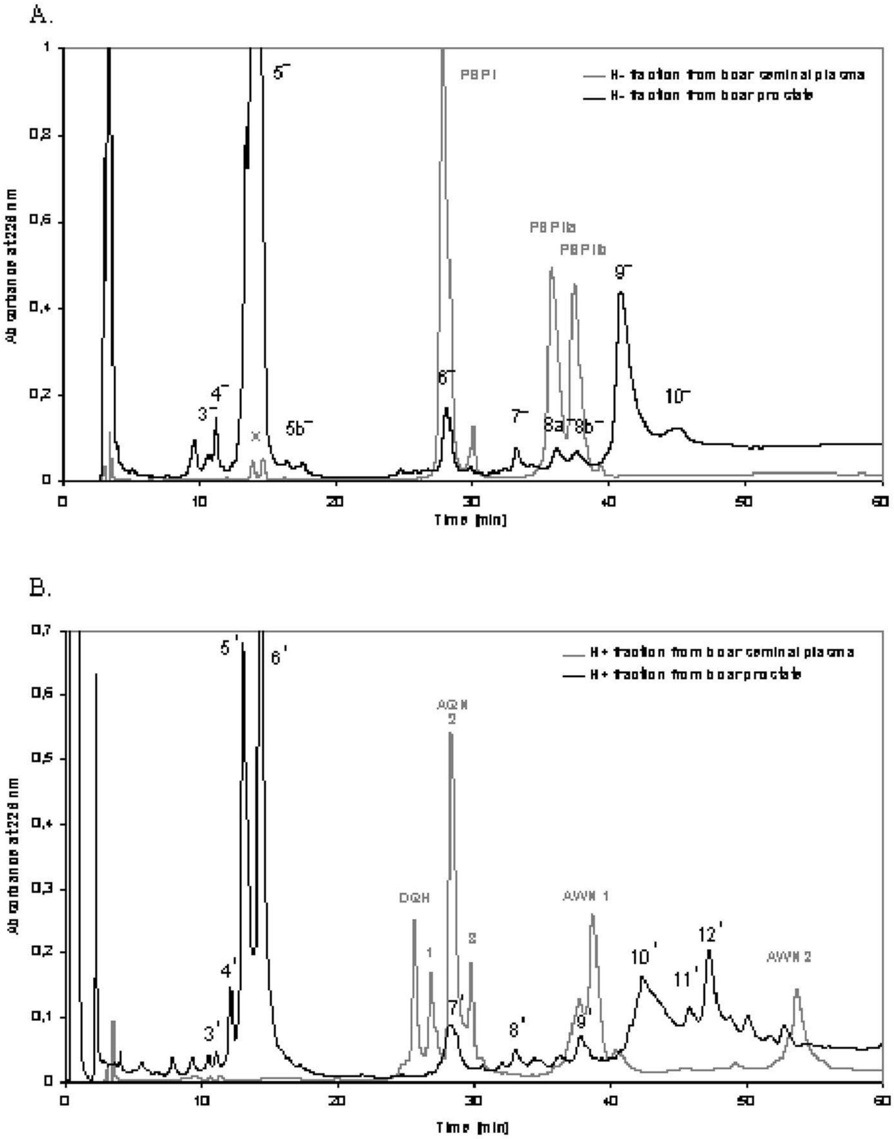

Fig. 2. RP HPLC of non-heparin-binding (A) and heparin-binding (B) fractions from boar seminal plasma and from boar prostate secretion. DQH, sperm surface protein; AQN, AWN, PSP, spermadhesins; x, β-microseminoprotein; black numbers, protein fractions from prostate secretion.

seminal plasma were compared by their RP HPLC profiles (Fig. 2A and B). The components of H^- and H^+ fractions from seminal plasma were identified previously [2,18]. The H^- fraction of seminal plasma contained mainly PSP I and II spermadhesins; DQH sperm surface protein, AQN and AWN spermadhesins were the main proteins of the H^+ fraction [18]. In H^- and H^+ fractions of boar prostate secretion, components corresponding to major proteins of H^- and H^+ fractions of seminal plasma were found in an apparently lesser amount than in seminal plasma fractions. Major proteins of both H^- (5^-) and H^+ (4^+– 6^+) fractions of prostate secretion corresponded by their retention times to β-microseminoprotein. β-Microseminoprotein was detected only in a negligible amount in the H^- fraction of seminal plasma.

SDS-electrophoreograms of RP HPLC-separated components of H^- and H^+ fractions of prostate secretion are shown in Fig. 3. Reduced and non-reduced samples yielded identical bands, with the exception of fractions 5^- (Fig. 3A) and 4^+–6^+ (Fig. 3B): bands of M_w 20 000 under non-reducing conditions and of M_w 10 000 after reduction. When subjected to amino acid sequencing analysis, no free N-terminal amino acid was determined in fractions 5^- and 6^+.

The results of SDS electrophoresis of protein components of H^- and H^+ fractions from seminal plasma and from prostate secretion with the same HPLC retention times were compared (Fig. 4A and B): PSP I, PSP IIa and PSP IIb with protein components 6^-, $8a^-$ and $8b^-$ from the H^- prostate fraction, respectively (Fig. 4A); similarly, AQN 2 and AWN 1 with protein components of the H^+ prostate fraction (7^+ and 9^+) (Fig. 4B). Multiplicities of zones of PSP I, PSP II, AQN 2 and AWN 1 proteins were the result of different glycosylation: all isoforms of particular proteins had the same N-terminal amino acid sequence [2,18]. Proteins isolated from prostate secretion exhibited similar multiplicities (Fig. 4).

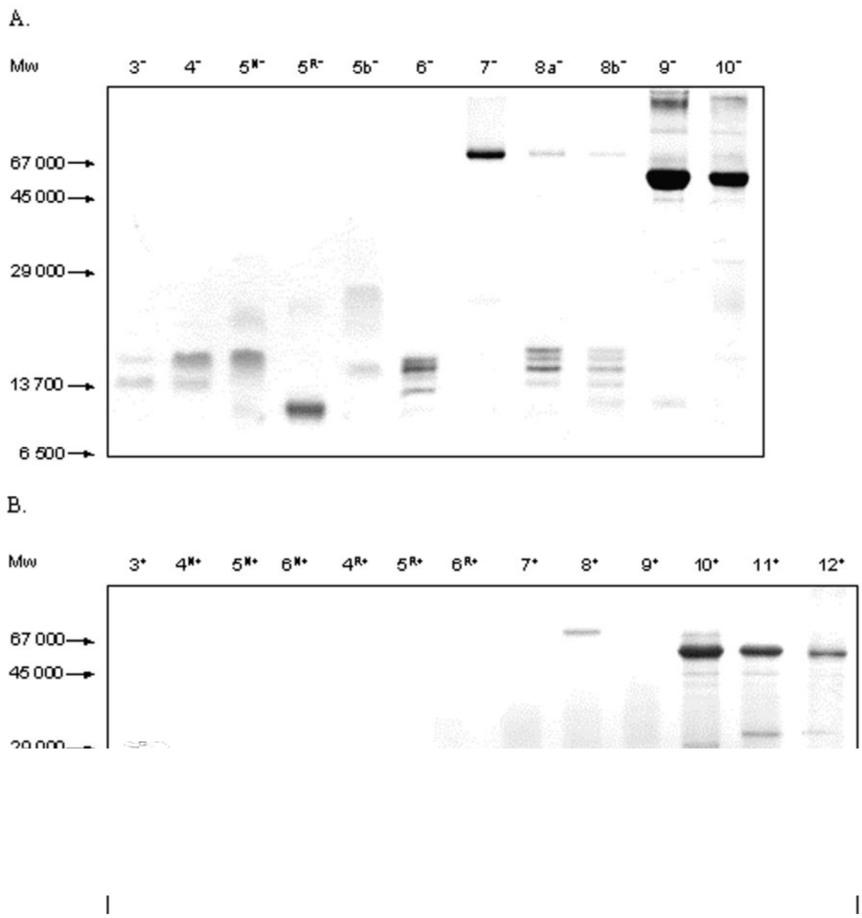

Fig. 3. 15% SDS–PAGE of HPLC-separated proteins from boar prostate: H^- fraction (A), H^+ fraction (B). N, non-reducing conditions; R, reducing conditions.