ACTA ABP BIOCHIMICA POLONICA

Vol. 60, No 2/2013
255–258
on-line at: www.actabp.pl

Communication

The antigenic character of zinc-binding proteins and their localization in boar reproductive tract — A preliminary study

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In this study immunoelectrophoretic and double immunodiffusion analyses were used to investigate the antigenic character of zinc-binding proteins (ZnBPs), whereas the indirect immunofluorescence technique was used to identify their origin in boar reproductive tract. The mmunoelectrophoretic analysis of ZnBPs of the seminal plasma resulted in the appearance of three antigenic protein complexes, while specific immunoreactivity patterns of the anti-ZnBP serum were detected by double immunodiffusion analysis. Indirect immunofluorescence technique confirmed that ZnBPs were secreted by different reproductive tract tissues, suggesting their contributions to the seminal plasma.

Key words: boar, seminal plasma, zinc-binding proteins, reproductive tract

Received: 08 February, 2013; revised: 25 April, 2013; accepted: 05 June, 2013; available on-line: 12 June, 2013

INTRODUCTION

Multifunctional proteins of the seminal plasma are implicated in different molecular processes, such as sperm capacitation, the acrosome reaction and sperm-oocyte interactions (Jonáková *et al.*, 2007; Rodriguez-Martinez *et al.*, 2011). Evidence has been shown that the seminal plasma proteins possess strong binding affinity for different substances, such as heparin and zinc (Arver & Eliasson, 1982, Hołody & Strzeżek, 1999, Manaskova & Jonakova, 2008, Vivacqua *et al.*, 2004, Strzeżek & Hopfer, 1987). However, more attention has been paid to zinc-binding proteins (ZnBPs) of the seminal plasma owing to their role in the biochemical and physiological events that occur during sperm-egg fertilization processes (Rodriguez-Martinez *et al.*, 2011, Henkel *et al.*, 2003).

Using affinity chromatography and electrophoretic techniques, ZnBPs have been isolated and characterized from canine and boar seminal plasma (Mogielnicka-Brzozowska *et al.*, 2011, Mogielnicka-Brzozowska *et al.*, 2012). In the boar ZnBPs have been shown to possess antibacterial, antioxidant and immunomodulating properties (Strzeżek & Hopfer, 1987, Boursnell *et al.*, 1975, Strzeżek *et al.*, 1987), and improved sperm motility and acrosome integrity, when added to extended semen stored at 4°C (Mogielnicka-Brzozowska *et al.*, 2011). In the literature, the source of ZnBPs of boar seminal plasma has not been clearly defined as yet. In this study immunoelectrophoretic and double immunodiffusion analyses were used to investigate the antigenic character of ZnBPs of boar reproductive tract, whereas the indirect immunofluorescence technique was used to identify the origin of ZnBPs in the seminal plasma.

MATERIALS AND METHODS

Fluid and tissues of the testes, epididymides, vesicular glands and prostate were collected from 2 Polish Large White adult boars (aged 3 years) at a local slaughterhouse and transported on ice to the laboratory. The epididymidal contents were obtained from incised cauda epididymidal ducts, using a syringe by aspiration, and were centrifuged (700 × *g*, 5 min at room temperature) to separate the fluid from the spermatozoa. The epididymidal fluid was further centrifuged (10 000 × *g*, 15 min at room temperature). The vesicular glands and prostate were dissected and the fluids collected by aspirations were centrifuged (10 000 × *g*, 15 min at room temperature). All the collected reproductive tract fluids were divided into aliquots and stored at −80°C, until required. Tissues of the reproductive tract organs (vesicular glands, prostate, caput, corpus and cauda epididymides, and testes) were dissected into small pieces, wrapped into aluminum foil, snap-frozen and stored in liquid nitrogen (−196°C), until required. All experimental procedures were performed in accordance with the approved guidelines of the Local Ethics Committee for Experimentation with Animals.

The ZnBPs have been purified from boar seminal plasma (Mogielnicka-Brzozowska *et al.*, 2011) and were used to prepare the rabbit antisera. Two rabbits were administered subcutaneous injections of 2 mg lyophilized ZnBPs, dissolved in 1 ml of phosphate buffer saline (PBS) emulsified with 1 ml of Freund's complete adjuvant. The animals were inoculated twice with 2 mg ZnBPs, dissolved in 1 ml of PBS emulsified with 1 ml of Freund's incomplete adjuvant, at intervals of 2 weeks after the first injection. Two weeks after the last inoculation, the rabbits were bled through the ear vein and the blood sera were tested for anti-ZnBP serum activity. Control serum was obtained from rabbits immunized with Freund's complete adjuvant, without the addition of ZnBPs.

The ZnBPs and seminal plasma were subjected to immunoelectrophoretic analysis, as previously described (Scheideggar, 1955). Following the polymerization of the agarose gel, longitudinal grooves were pre-

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Abbreviations: ZnBPs, zinc-binding proteins form boar seminal plasma

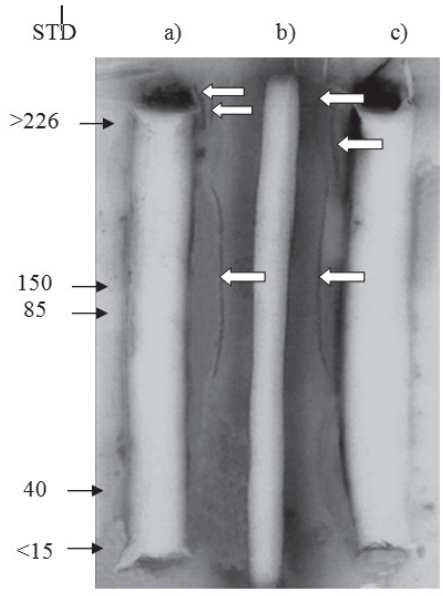


Figure 1. Immunoelectrophoretic analysis of anti-ZnBP rabbit serum with ZnBPs of boar seminal plasma:
(**a**) isolated ZnBPs, (**b**) anti-ZnBP serum and (**c**) seminal plasma. The isolated ZnBPs and seminal plasma showed three precipitation arcs, as indicated by the white arrows. The molecular masses of native ZnBPs were determined by gel filtration chromatography (Mogielnicka-Brzozowska *et al.,* 2011). STD, standard molecular masses of ZnBPs.

pared and filled with 500 µl of anti-ZnBP serum (10 mg antibody/ml) and the control serum. The acquired antigen protein complexes were analyzed following the incubation of the prepared gels for 24 h at 37°C in a humid chamber. Gel filtration chromatography was used to determine the molecular masses of the ZnBPs (Mogielnicka-Brzozowska *et al.*, 2011).

Fluids of the vesicular glands, prostate and cauda epididymidis, and the seminal plasma were subjected to double immunodiffusion analysis, as described in a previous study (Ouchterlony, 1963). Round wells were prepared in the Petri's plates following the complete polymerization of the agarose gel. The rabbit anti-ZnBP serum (10 mg antibody/ml) was applied to the central well of the plate, whereas the reproductive tract fluids and seminal plasma were applied to the surrounding wells. Samples were incubated for 48 h at room temperature in a humid chamber and analyzed for the appearance of precipitation lines.

Tissues of the vesicular glands, prostate, epididymides (caput, corpus, cauda) and testes were fixed, cleaned, embedded in gelatin, and sectioned at 5 µm, using a freezing microtome (Freezing Microtome, Richert Yung, Cryocat 1800, Leica Instruments, Nussloch GmbH, Germany). Aliquots of the tissue sections were overlaid for 30 min at room temperature with anti-ZnBP serum (1 mg antibody/ml) or with the control serum. Following the incubation, the slides were washed 3' times with PBS and overlaid for 1 h at room temperature with a second antibody goat anti-rabbit IgG FITC conjugate, 1:80 (Sigma, Aldrich, St. Louis, MO, USA). After washing with PBS, the sections were coated with 7% (v/v) glycerol. The labeled sections were analyzed and photographed with a Nikon Microphot FXA microscope, equipped with epifluorescence illumination.

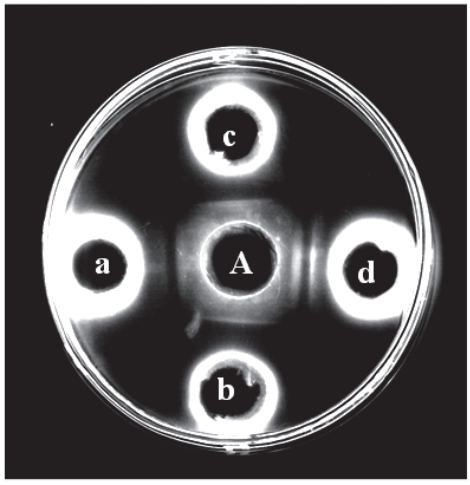


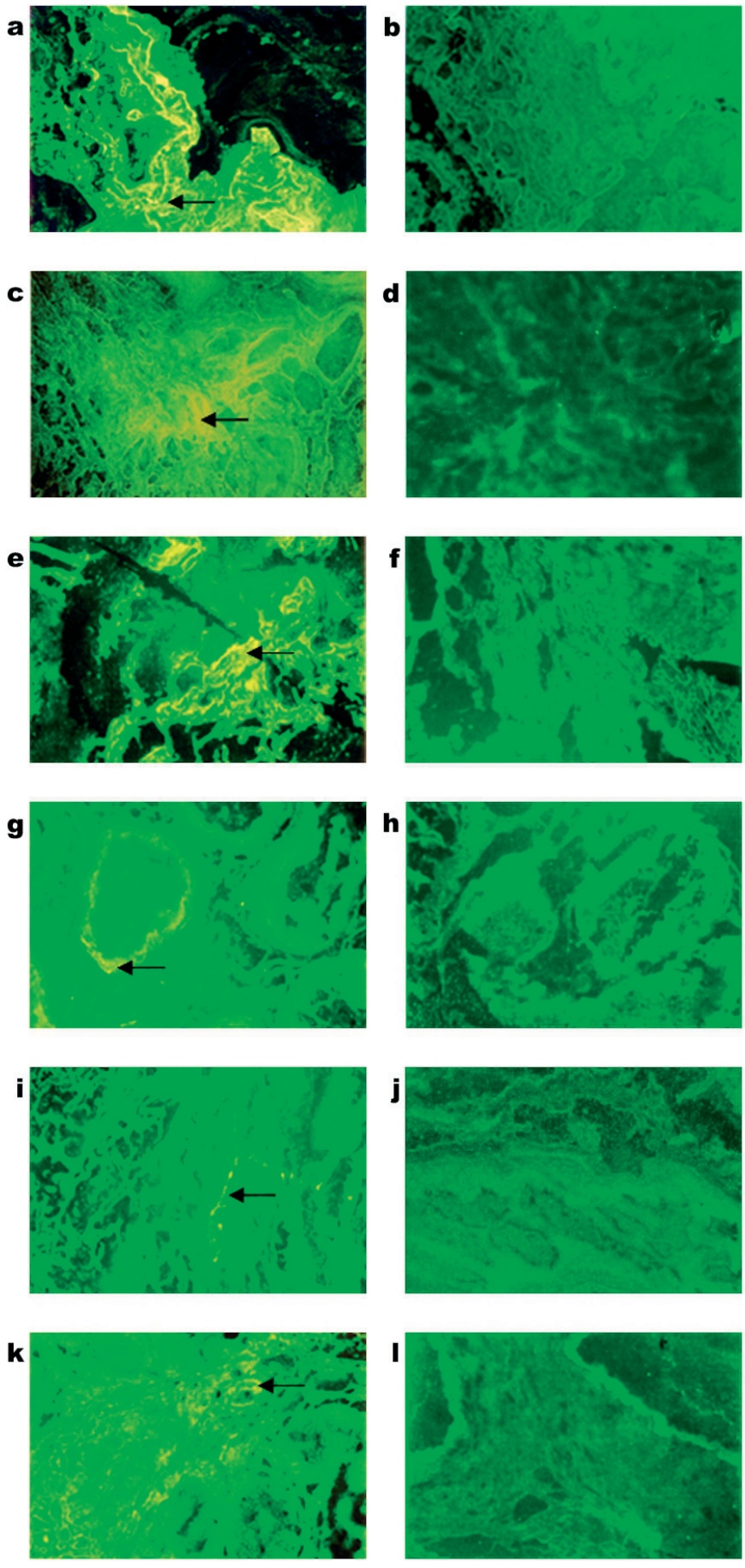
Figure. 2. Double immunodiffusion of anti-ZnBP rabbit serum with fluids of the (a) vesicular glands, (b) prostate, (c) cauda epididymidis, and (d) the seminal plasma.
A strong and a weak line of precipitation were visible in the vesicular gland fluid. Both prostate and cauda epididymidal fluids displayed a strong line of precipitation, whereas two strong precipitation lines occurred in the seminal plasma. The anti-ZnBPs rabbit serum was applied to the central well (**A**). White precipitation lines represent the reaction of the anti-ZnBP rabbit serum with ZnBPs.

RESULTS AND DISCUSSION

In this study, immunoelectrophoretic analysis was performed to analyze more precisely the antigenic components of the ZnBPs of boar seminal plasma, which originate in different reproductive tract secretions. The immunoelectrophoretic analysis of the isolated ZnBPs (Fig. 1a) and seminal plasma components (Fig. 1c) with anti-ZnBP serum revealed three antigenic protein complexes, identified as three precipitation arcs. It was found that the molecular masses of the antigenic protein complexes ranged from 85 kDa to more than 226 kDa, as analyzed by gel filtration chromatography (Fig. 1). It seems that the antigenic character of seminal plasma ZnBPs reflects their participation in immunological reactions associated with sperm-egg fertilization processes in the female reproductive tract. Evidence has been shown that boar seminal plasma components might be involved in the regulation of the uterine immune activity (Veselsky *et al.*, 2002).

Double immunodiffusion analysis showed the immunoprecipitation reaction patterns of anti-ZnBP serum for different fluids of the reproductive tract and seminal plasma (Fig. 2). The anti-ZnBP serum gave a strong and a weak line of precipitation against the protein components of the vesicular gland fluid (Fig. 2a), whereas two strong precipitation lines occurred in the seminal

Figure 3. Indirect immunofluorescence of seminal plasma zinc-binding proteins (ZnBPs) in different secretory tissues of boar reproductive tract:
(**a**) vesicular gland, (**c**) prostate, (**e**) caput epididymidis, (**g**) corpus epididymidis, (**i**) cauda epididymidis, and (**k**) testis, with the respective negative serum controls (**b, d, f, h, j** and **l**). The green-yellow fluorescence represents the reaction of the anti-ZnBP rabbit serum with ZnBPs. The bright yellow fluorescence, indicated by the arrows, represents high concentration of ZnBPs in the tissues. All images were taken at 200 x magnification.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plasma (Fig. 2d). By contrast, the anti-ZnBP serum gave one precipitation line against the fluids of the prostate (Fig. 2b) and cauda epididymidis (Fig. 2c). Such findings reflect the specific patterns of immunoreactivity of the anti-ZnBP serum to the seminal plasma and the reproductive tract organs.

Indirect immunofluorescence staining of all the tissue sections gave positive reactions when incubated with rabbit anti-ZnBP serum (Fig. 3). However, the strongest immunofluorescence tissue staining was observed in the vesicular glands (Fig. 3a), suggesting that these glands contribute a major portion of ZnBPs to the seminal plasma compared with the prostate (Fig. 3c), epididymides (Fig. 3e, 3g and 3i) and testes (Fig. 3k). These findings are in accordance with those of a previous study indicating that boar vesicular glands might play a key role in the secretion of ZnBPs to the seminal plasma (Boursnell *et al*., 1975, Strzeżek *et al*., 1987). In bulls, significant amounts of ZnBPs have been detected in the cauda epididymidis and vas deferens (Henkel *et al*., 2003), whereas in the dog the prostate seems to contribute the bulk of the ZnBPs to the seminal plasma (Mogielnicka-Brzozowska *et al*., 2012). Moreover, in humans most of the seminal plasma proteins showing zinc-binding capacity originate in the vesicular glands and prostate (Arver & Eliasson, 1982, Vivacqua *et al*., 2004). Findings of the current study and those of other studies emphasize the species-specific variations in the reproductive tract contributions of seminal plasma ZnBPs, which could be related to their biological functions.

The protein composition of ZnBPs of boar seminal plasma is very complex. It is noteworthy that ZnBPs of boar seminal plasma occur in their native states, as high-molecular-weight aggregates (Mogielnicka-Brzozowska *et al*., 2011) and are believed to belong to the family of spermadhesins (Hołody & Strzeżek, 1999), which are implicated in many functional properties of spermatozoa (Jonáková *et al*., 2007, Rodriguez-Martinez *et al*., 2011). The findings of this study provide new insights into the origin of ZnBPs of boar seminal plasma. However, more studies are needed to provide a greater understanding of the biological significance of ZnBPs, originating in different secretions of boar reproductive tract. Furthermore, the applications of different laboratory techniques such as Western blot analysis and reverse transcriptase-polymerase chain reaction (RT-PCR) method, are needed to verify ZnBP mRNA expression in different tissues.

Acknowledgements

This study was supported by a KBN grant 2P06D 00128 and funds (No. 0103.0803) from the University of Warmia and Mazury in Olsztyn.

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