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Original article

Isolation and characterization of zinc-binding proteins of canine seminal plasma

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Abstract

Zinc-binding proteins from seminal plasma (ZnBPs) originate in the secretions of different accessory sex glands and are implicated in key events associated with sperm-egg fertilization processes. This study describes the isolation and characterization of the ZnBPs of canine seminal plasma. Ejaculates were collected from three crossbred dogs for a 2-week period. The ZnBPs as well as non zinc-binding proteins (nZnBPs) were isolated by zinc-dependent affinity chromatography. The isolated fractions were subjected to native gel electrophoresis (one-dimensional polyacryamide gel electrophoresis, PAGE) and sodium dodecyl sulphate polyacryamide gel electrophoresis (SDS-PAGE), using denaturing and reducing conditions. Zinc-elution profile using affinity chromatography displayed two protein fractions represented by the nZnBPs and ZnBPs, respectively. Using native gel electrophoresis, it was found that both the nZnBPs and ZnBPs occurred in their native state as aggregates, ranging from 140 to 669 kDa. The nZnBPs were disaggregated into 8 protein bands, with molecular weights ranging from 10.7 to 79.7 kDa, following SDS-PAGE analysis. By contrast, SDS-PAGE analysis of the ZnBPs revealed 13 protein bands, with molecular weights ranging from 11.6 to 152.3 kDa. Densitometric analysis showed that 46-48% of nZnBPs could be accounted by protein fractions with molecular weights of 10.7 and 14.2 kDa. Also, 2 protein fractions with molecular weights of 11.6 and 14.3 kDa, were predominant in ZnBPs, accounting for approximately 28-30% of the total proteins. These results demonstrate the zinc-binding capacity of proteins secreted by the canine prostate. The findings of this study indicate that ZnBPs of canine seminal plasma comprise several protein fractions, which might be implicated in the reproductive processes in the dog.

Key words: dogs, seminal plasma, electrophoresis, zinc-binding proteins

Introduction

Seminal plasma, a complex mixture of secretions originating from the testis, epididymis and accessory sex glands, modulates the fertilizing ability of spermatozoa (Russell et al. 1984, Strzeżek et al. 2005). Increasing evidence shows that some of the seminal plasma proteins are bound to the sperm surface during ejaculation and thus forming protein-coating layers (Jonáková et al. 2007). As regards the dog, most of the seminal plasma components, which are implicated in the sperm function, originate in the prostatic secretion (Nothling et al. 1993, Sirivaidyapong et al. 2001, De Souza et al. 2006, 2007, Strzeżek and Fraser 2009, Cheema et al. 2011). Electrophoretic analysis has shown that proteins, secreted

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by the canine prostate, are different from the accessory sex gland secretions of other animal species, in terms of both quantity and quality (Dubiel 1974, Dubé et al. 1985).

Accumulating evidence has been shown that zinc ions, secreted by the prostate and vesicular glands, bind to different proteins ligands, which are implicated in sperm-egg fertilization processes in the female reproductive tract (Boursnell et al. 1975, Arver 1982, Hołody and Strzeżek 1999, Siciliano et al. 2000, Mogielnicka-Brzozowska et al. 2011). Zinc-binding proteins (ZnBPs) have been identified in the seminal plasma of human (Arver 1982, Arver and Eliasson 1982, Siciliano et al. 2000) and boar (Boursnell et al. 1975, Strzeżek et al. 1987, Hołody and Strzeżek 1999, Mogielnicka-Brzozowska et al. 2011). Evidence has been shown that most of the zinc in human and canine seminal plasma originates in the prostate (Johnson et al. 1969, Lindholmer and Glaumann 1972). Zinc is involved in normal testicular development, spermatogenesis, and sperm function (Henkel et al. 2003). It has been demonstrated that approximately 93% of the zinc is located in the flagellum of the ejaculated spermatozoa (Henkel et al. 1999), while the remaining 7% zinc is located in the sperm head, contributing to the stability of the quaternary structure of the sperm nuclear chromatin (Björndahl and Kvist 1990, Kvist et al. 1990).

To date, ZnBPs of canine seminal plasma have not been investigated although the presence of heparin-binding proteins have been reported (De Souza et al. 2006). The aim of this study was to identify and characterize the ZnBPs of canine seminal plasma, using zinc-dependent affinity chromatography and electrophoretic analysis. Also, the potential role of the ZnBPs in the reproductive processes in the dog has been addressed.

Materials and Methods

Animals and semen collections

Three healthy crossbred dogs (aged 3 to 5 years) of unknown fertility were used in this study. Ejaculates (n = 6) were collected from each dog in November for a 2-week period, using digital manipulation. The animals were kept in individual cages with access to water (*at libitum*) and fed with commercial canine food. All experiments were carried out in accordance with the guidelines set out by the Local Ethics Committee for Experimentation with Animals.

Each ejaculate was centrifuged (1000 × g, 15 min at room temperature) to remove the suspended spermatozoa. The recovered seminal plasma was further centrifuged (10000 × g, 10 min at room temperature). The resultant supernatant was dialyzed against deionized H_2O for 24h at room temperature and stored at -80°C, until required for further analysis.

All chemical reagents were of the highest purity grade available and were purchased from Sigma Aldrich (St. Louis, MO, USA), unless otherwise stated.

Protein determination

Total protein content was measured in the fresh seminal plasma and in the recovered protein fractions obtained by zinc-dependent affinity chromatography, according to Lowry et al. (1951), using bovine serum albumin (BSA, Serum and Vaccine Production, Cracow, Poland) as a standard.

Zinc-dependent affinity chromatography

Zinc-dependent affinity chromatography was used to isolate the non zinc-binding (nZnBPs) and zinc-binding proteins (ZnBPs) of pooled seminal plasma of 3 dogs, as described previously (Hołody and Strzeżek 1999, Mogielnicka-Brzozowska et al. 2011), with some modifications. Chelating Sepharose Fast Flow gel, packed in 10-ml column, HR 10/10 (Amersham Biosciences), was equilibrated with 0.5 M NaCl in 0.5 M Tris-HCl buffer (pH 8.0) and saturated with an aqueous solution of 2% $ZnCl_2$. After the column had been washed twice with the equilibration buffer, aliquots (2 ml) of diluted seminal plasma with equilibration buffer (1:1) were loaded. The column was washed to remove unabsorbed proteins – non zinc-binding proteins (nZnBPs). The absorbed zinc-binding proteins (ZnBPs) were eluted with a buffer comprising 0.5 M Tris-HCl buffer (pH 7.5) and

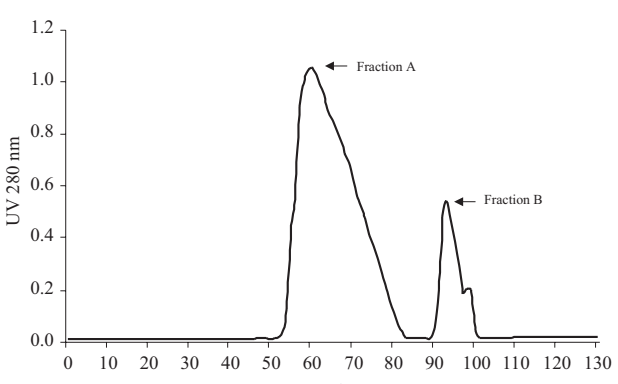

Fig. 1. Graphic image plotted by UNICORN Control System software (Amersham Biosciences, Uppsala, Sweden) of (A) non-zinc-binding (nZnBPs) and (B) zinc-binding proteins (ZnBPs) of canine seminal plasma separated by zinc-dependent affinity chromatography.

0.1 M imidazole. Both the nZnBPs and ZnBPs fractions were eluted in 5-ml fractions. The column was regenerated with 0.5 M Tris-acetate, 0.5 M NaCl and 0.5 M EDTA (pH 7.5). The data and graphics of the isolated nZnBP and ZnBP fractions were plotted by specialized software (UNICORN™, Amersham Biosciences, Uppsala, Sweden). Protein-containing fractions were collected and pooled (Fig. 1). The recovered nZnBP (Fraction A) and ZnBP (Fraction B) fractions were dialyzed against deionized H_2O for 24h at room temperature. The selected proteins fractions were analyzed for protein content and stored at – 80°C until further analysis.

Electrophoretic characterization of ZnBPs

One dimensional polyacrylamide gel electrophoresis, under non-denaturing (PAGE), and denaturing and reducing (SDS-PAGE) conditions, were performed according to the method of Laemmli (1970).

For PAGE analysis, aliquot of protein samples were diluted (1:1) with 2-fold concentrated buffer containing 0.34 M Tris-HCl, 20% glycerol, 2% bromophenol blue (pH 6.8). Native electrophoresis was conducted in 6% polyacrylamide gel slabs. Electrophoresis was performed at constant voltage (120V) in a buffer (0.5 M Tris, 0.25 M glycine, pH 8.3). After electrophoresis the gel was stained with Coomassie Brilliant Blue. Molecular mass was estimated using the High Molecular Weight Calibration Kit for Native Electrophoresis (Amersham Biosciences).

Briefly for SDS-PAGE analysis, aliquots of protein samples were diluted at ratio (1:1) with 2-fold concentrated lysis buffer (0.34 M Tris-HCl, 10% SDS, 20% glycerol, 2% β-mercaptoethanol, 2% bromophenol blue, pH 6.8) and heated for 5 min at 95°C. Before loading, the samples were centrifuged at (10 000 × g for 5 min at room temperature) and the supernatant was used for electrophoretic analysis. Different protein concentrations (35, 17 and 4 µg) were loaded into the gel wells to obtain optimal resolution of the analyzed protein fractions. Electrophoresis was performed in 12% polyacrylamide gel in a buffer containing 0.5 M Tris, 0.25 M glycine and 0.5% SDS (pH 8.3) at a constant voltage (120V). Following electrophoresis, the gel was stained with Coomassie Brilliant Blue and the molecular mass of the protein bands were determined using the Low Molecular Weight Standards (Amersham Biosciences). Densitometric analysis was performed to quantify the protein bands, using the MultiAnalyst software (BioRad, Warsaw, Poland).

Results

The overall mean total protein content in the pooled seminal plasma of the 3 dogs was 29.9 ± 1.8 mg/ml (mean ± SD).

Figure 1 shows the graphic image plotted on the zinc-dependent affinity chromatography of eluted canine seminal plasma proteins. The zinc-elution profile showed 2 different peaks of the isolated seminal plasma protein fractions; the higher curve was represented by nZnBPs (Fraction A), whereas the lower curve contained the ZnBPs (Fraction B). Image software analysis showed that the ZnBPs accounted for 4 to 10% of the total seminal plasma proteins.

Electrophoresis analysis (PAGE) of the peak-containing the nZnBPs and ZnBPs of canine seminal plasma showed that these proteins occurred in their native state, as high molecular weight aggregates, ranging from 140 to 669 kDa (Fig. 2A for the nZnBPs and Fig. 2B for the ZnBPs).

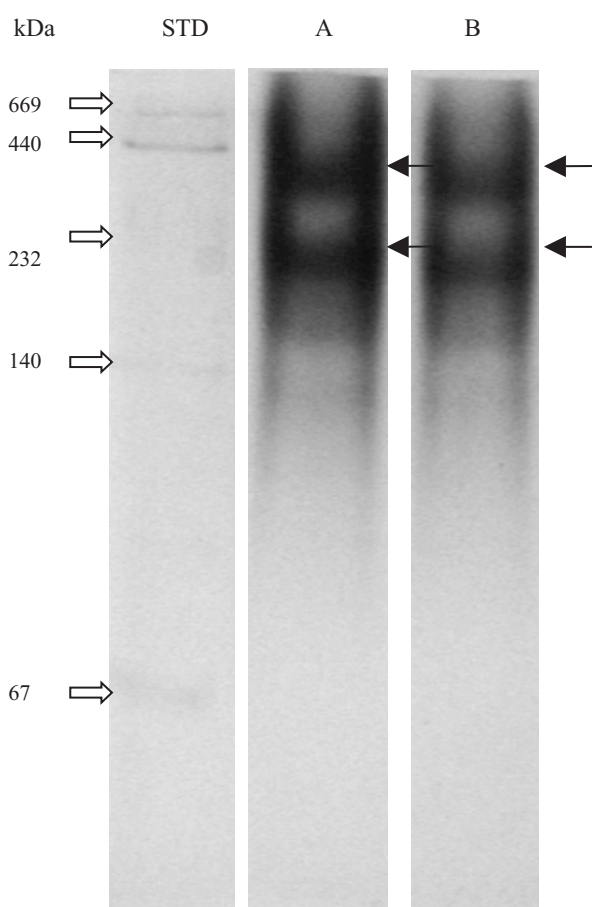

Fig. 2. Polyacrylamide gel electrophoresis (PAGE) of isolated (A) non-zinc-binding (nZnBPs) and (B) zinc-binding proteins (ZnBPs) of canine seminal plasma in their native states. Each lane consists of 122 µg of protein. STD – High Molecular Weight Calibration Kit for Native Electrophoresis (Amersham Biosciences).

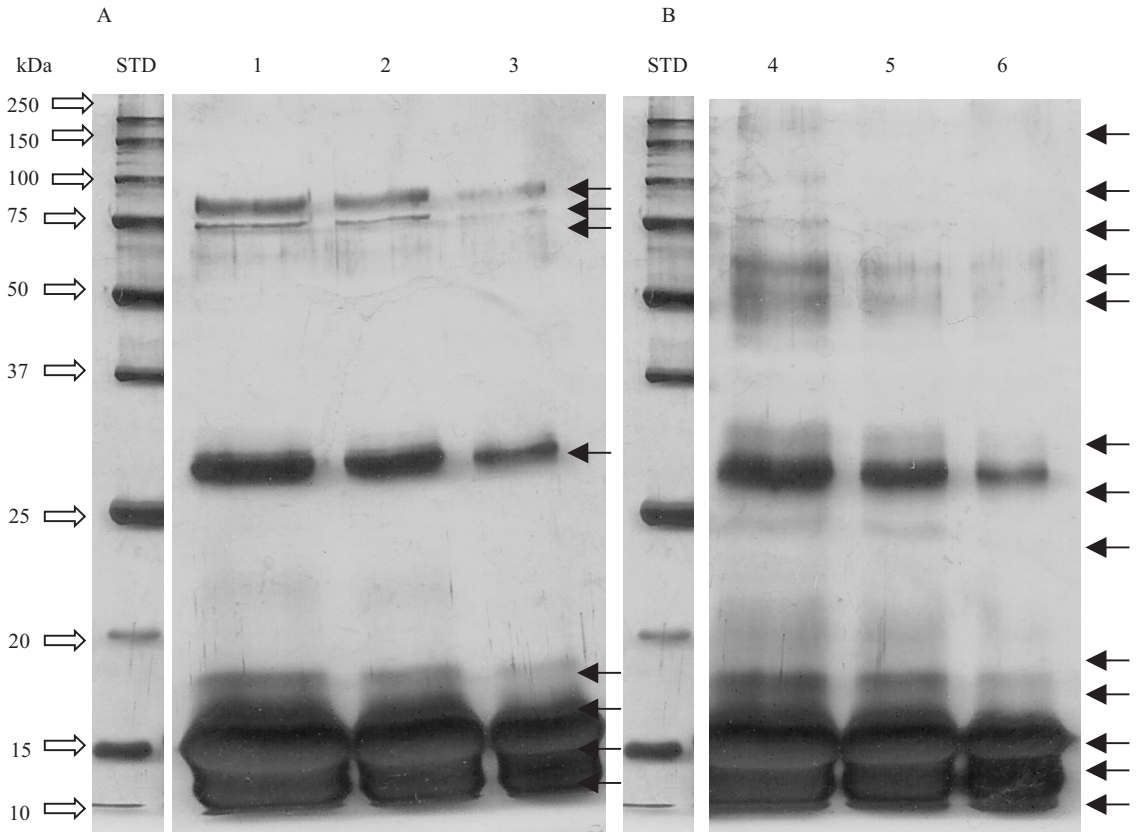

Fig. 3. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of isolated (A) non-zinc-binding (nZnBPs) and (B) zinc-binding proteins (ZnBPs) of canine seminal plasma. Different amounts of protein were loaded into the gel wells to obtain optimal resolution of the protein bands. Lanes 1 and 4: 35 µg. Lanes 2 and 5: 17 µg. Lanes 3 and 6: 4 µg. STD – Precision Plus Protein Standard (BioRad).

Under denaturing and reducing conditions (SDS-PAGE), a total of 21 protein bands were identified in both the nZnBPs and ZnBPs of the pooled seminal plasma (Fig. 3). Furthermore, SDS-PAGE analysis revealed that the nZnBPs were disaggregated into 8 protein bands, with molecular weights of 10.7, 14.2, 16.5, 17.8, 27.6, 63.4, 69.3 and 79.7 kDa (Fig. 3A). By contrast, SDS-PAGE analysis of the eluted ZnBPs revealed 13 protein bands, with molecular weights of 11.6, 14.3, 16.2, 18.8, 20.1, 24.3, 27.6, 29.8, 50.1, 60.2, 70.2, 111.4 and 152.3 kDa (Fig. 3B). Densitometric analysis of the SDS-PAGE gel showed that 2 protein fractions, with molecular weights of 10.7 and 14.2 kDa, accounted for approximately 46-48% of the total proteins detected in the nZnBPs. On the other hand, protein fractions, with molecular weights of 11.6 and 14.3 kDa, were the predominant in the ZnBPs, accounting for approximately 28-30% of the total proteins.

Discussion

To the best of our knowledge, this study has isolated and characterized, for the first time, the zinc-ligandprotein complexes of canine seminal plasma. Our findings have confirmed that zinc of canine seminal plasma has a strong affinity for protein ligands with different molecular weights. Moreover, it was shown that zinc could bind to high, intermediate and low molecular weight protein ligands, originating in the vesicular and prostatic secretions in human seminal plasma (Arver 1982, Arver and Eliasson 1982, Siciliano et al. 2000). These authors have postulated that a redistribution of zinc takes place at ejaculation. In the present study, PAGE analysis of the ZnBPs of canine seminal plasma detected aggregates in their native state. Since most of the secretions of canine seminal plasma are derived from the prostate (Dube et al. 1985, Nothling et al. 1993), it can be suggested that the bulk of the proteins showing zinc-binding capacity originate mainly in the prostate. Also, studies in our laboratory have confirmed that zinc of boar seminal plasma can interact with protein ligands, high-molecular weight components, in their native state at ejaculation (Strzeżek et al. 1987, Hołody and Strzeżek 1999, Mogielnicka-Brzozowska et al. 2011).

Evidence has been shown that the interactions of zinc of the seminal plasma with high-molecular

weight ligands of the seminal plasma can affect the biological property of zinc, and consequently sperm function (Björndahl and Kvist 1990, Kvist et al. 1990). According to Björndahl and Kvist (1990), protein ligands of the seminal plasma bind the free and loosely bound zinc at ejaculation, reducing the zinc fraction available for the sperm cells. Moreover, high abundance of the protein ligands could deplete zinc from spermatozoa, resulting in impaired sperm function (Kvist et al. 1990). In the present study, SDS-PAGE analysis showed that the ZnBPs of canine seminal plasma disaggregated into several protein fractions with various molecular weights, which can have various effects on sperm function. These results have been corroborated by findings of other authors, when ZnBPs of human and boar seminal plasma were analyzed by SDS-PAGE (Arver 1982, Arver and Eliasson 1982, Hołody and Strzeżek 1999, Jonhkovh et al. 2007).

In recent years, several proteins, which have been isolated and characterized in canine seminal plasma, have been shown to be associated with fertility (Dubiel 1974, De Souza et al. 2007, Cheema et al. 2011). Several authors have reported that components of prostatic secretion can have various effects on sperm function following semen storage or cryopreservation (Nothling et al. 1993, Sirivaidyapong et al. 2001, Strzeżek and Fraser 2009). Moreover, significant correlations of two seminal plasma proteins (67 and 58.6 kDa) were found with sperm quality characteristics, and suggested to be valuable markers for fertility (De Souza et al. 2007). It was demonstrated that the concentrations of 4 protein fractions (14, 24, 70 and 82 kDa) of canine seminal plasma correlated with semen quality parameters (Cheema et al. 2011). Recently, it has been reported that proteins of intermediate- and low-molecular weights (ranging from 24 to 42 kDa) account for 61.3 to 74.3% of the total protein concentration in the seminal plasma of dogs (Cheema et al. 2011). Several authors reported that 12-14 kDa and 15 kDa protein fractions were enzyme arginine esterase, which could account for about 90% of the total proteins secreted by the prostate and 30% of the seminal plasma proteins in the dog (Isaacs and Coffey 1984, Dube et al. 1985, De Souza et al. 2007). In our recent study, we have shown that the ZnBPs of boar seminal plasma improved sperm motility and acrosome integrity of preserved spermatozoa, presumably by coating the sperm surface (Mogielnicka-Brzozowska et al. 2011). Similar findings have been reported by other studies (Nothling et al. 1993, Sirivaidyapong et al. 2001, Strzeżek and Fraser 2009), indicating that the prostatic secretion possesses sperm-coating substances, which protect sperm function during semen preservation. Our results have shown that ZnBPs comprise 4-10% of the total seminal plasma proteins, and under denaturing and reducing conditions, disaggregated into 13 protein fractions, with molecular weights ranging from 11.6 to 152.3 kDa. It is possible that the ZnBPs of canine seminal plasma, particularly the predominant low-molecular weights protein components (11.6 and 14.6 kDa), might be implicated in key events associated with sperm-egg fertilization processes. This assumption has been confirmed by findings of several authors (Töpfer-Petersen 1999, Strzeżek et al. 2005, De Souza et al. 2006, Jonáková et al. 2007), indicating that seminal plasma proteins bound on the sperm surface are implicated in sperm-egg fertilization processes, including sperm capacitation and egg recognition, in the female reproductive tract. On-going studies in our laboratory have shown that ZnBPs comprise the enzyme arginine esterase subunits and have beneficial effects on sperm plasma membrane integrity during liquid storage of canine semen (unpublished data). Besides the dog, seminal plasma proteins have been suggested to be good candidates for markers of fertility in the horse, bull and boar (Killian et al. 1993, Brandon et al. 1999, Nowak et al. 2010).

In summary, the findings of this study indicate that the ZnBPs of canine seminal plasma comprise several protein fractions, which might be implicated in key events associated with the reproductive processes in the dog. Further studies on the characterization of the ZnBPs of canine seminal plasma and their binding property are currently underway in our laboratory. It is envisaged that the ZnBPs will be a valuable marker for canine sperm fertility and will be useful in the improvement of the reproductive technologies in the dog.

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