Review Article

Proteases and Proteases Inhibitors of Semen – A Review

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Abstract | The ejaculate composition is extremely complicated and variable among livestock species. The seminal proteins and enzymes are vital for ejaculate metabolism, spermatozoa performance, survival, and transport within the feminine reproductive tract. Proteases and proteinase inhibitors are secreted by the accessory sex glands of the male reproductive tract and find mixed with the spermatozoa throughout ejaculation. But an entire understanding of those enzymes and their performance in numerous class species is not obtainable. The performance of the assorted proteinases and protease inhibitors of ejaculate stay a mystery, and only a few studies are conducted concerning the characterization of those enzymes. Throughout this review, we tend to target the current understanding of the key proteases and their inhibitors of ejaculate from various mammalian species.

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INTRODUCTION

Semen, a liquid cellular suspension comprising of spermatozoa, the male germ cell and accessory secretory product of the male procreative tract. Numerous enzymes as well as proteolytic enzymes and protease inhibitors are associated with seminal plasma. These are secreted by the accessory sex glands of the male reproductive tract and these were found mixed with the gamete throughout ejaculation. The precise functions of proteolytic enzymes and protease inhibitors of seminal plasma are presently unknown. Some of these proteases have been related to fertility of ejaculate. In human, proteases and their regulators are reportable to play role in gamete storage, maturation, activation and ejaculate activity (Propping et al, 1978). Recently, numerous proteolytic enzymes and protease inhibitors have conjointly been detected in bovine, buffalo (Gurupriya et al., 2014), turkey and boar (Metayer et al., 2002) ejaculate. The changes within the activities of proteases and their regulators and their characterization are less studied in livestock species. Therefore, this review focuses on the main proteases and their inhibitors of ejaculate of various classes of livestock species.

Spermatozoa

Sperm cells are produced within the testes by a complex process of spermatogenesis in which transformation of male germ cells (spermatogonia) to mature spermatozoa occurs. The duration of seminiferous epithelial cycle and spermatogenesis for buffalo spermatozoa is 8.6 and 38 days respectively. The daily sperm

production rate is estimated as 4.04 x 10^9 spermatozoa (Sharma and Gupta, 1979).

Sperm metabolize molecules, mainly sugars and their derivatives (e.g. fructose, glucose, mannose and pyruvate), mainly by aerobic and anaerobic pathways, to produce energy for motility and also the maintenance of ionic gradients across membranes. Forward motility of sperm cell results from controlled and coordinated waves of flagella bending movements progressing from neck throughout the length of the sperm tail. The sperm after being synthesized in the testes are delivered into epididymis for concentrating sperm population to a density of about 4x10^6/mm^3 and maturation of sperm during its journey from head to tail of epididymis and provides nourishment to sperm for its storage (Ganguly, 1979).

Spermatozoa originate from seminiferous tubules of the testes and are suspended in seminal plasma. The fully matured spermatozoa consist of a flattened head having condensed nucleus and the overlying acrosome. Nucleus of sperm cell contains chromatin material. The spermatozoa are surrounded by semi-permeable plasma membrane that maintains the chemical ionic gradient and other components. The sperm is considered demeaned (deteriorated) and is not capable to fertilize in vivo if the plasma membrane is undiminished. Assessing the plasma membrane integrity of sperm is crucial for insemination.

Arosomal cap is situated between plasmalemma and anterior portion of sperm head. Acrosome is considered as a specialized form of lysosome, with acrosome cap at the anterior end and equatorial region posteriorly. Acrosin and hyaluronidase are the main two enzymes found to be associated with the acrosome (Morton, 1977). These acrosomal enzymes help in the diffusion of the cumulus oophorus and lysis of the zona pellucida. The equatorial region is enzymatically empty. The equatorial segment of acrosome and anterior portion of post-acrosomal region initially fuses with the oocyte membrane during fertilization. The acrosome must remain undiminished during the transport of the sperm to the isthmus of the fallopian tube until fertilization has achieved. This preliminary binding will boost-up the acrosome reaction which further releases acrosomal enzymes and its activation. This in association with the obtained hyper activated motility will trigger the sperm to penetrate zona pellucida (Holden et al., 1990). Premature acrosome reactions figure the sperm infertile, and therefore, assessment of acrosome integrity is very important for fertilization to occur. The middle piece of tail contains mitochondria which provide machinery for trapping and storing energy in the form of ATP, derived from respiration and glycolysis. Neck connects sperm head with its tail, which is subdivided into middle, principal and end pieces respectively. The tail portion is rich in phospholipids much of which is plasmalogens.

Mammalian spermatozoa do not exhibit fertilizing ability immediately after ejaculation and delivered in the female reproductive tract. The sperm should reach the ipsilateral side of the isthmus part of the oviduct where ovulation occurs (Rodriguez-Martinez et al., 2005). The spermatozoa undergo physiological change known as capacitation at this site (Austin, 1951 and Chang, 1951). This functional maturation of the spermatozoa is prerequisite for fertilization (Shimatsu et al., 1992). Capacitation involves many biochemical changes like hyper activated sperm motility and acrosome reaction (Wassarman, 1994). The hyperactive motile sperm shows whipping movements of the tail along with sideways swinging movements of the head and can thus easily penetrate the zona pellucida layer. The physiological change known as capacitation occurs within the female reproductive tract helps the sperm to penetrate and fertilize the egg (Austin, 1951). The associated changes like change in intracellular calcium concentration, protein phosphorylation, acrosomal matrix and membrane rearrangement (Garcia Herreros et al., 2005) and change in sperm motility also occurs. The molecular modifications like removal of spermatozoal-bound factors from seminal plasma and epididymal secretions, efflux of membrane cholesterol, change in membrane permeability, and increased influx of calcium ions (Cormier et al., 1997) also occurs. Both in vivo and in vitro (Ded et al., 2010) capacitated spermatozoa perform acrosome reaction after zona pellucida recognition.

The acrosome reaction is followed by various processes including remodeling and vesiculation of surface glycoprotein layer of outer acrosomal membrane. Imbalance between intracellular and extracellular calcium concentration results in exocytosis of the acrosome contents (Harrison and Roldan, 1990 and Jin et al., 2011) producing connecting pores results in leakage of acrosomal contents. Outer acrosomal membrane which acts as a coating of the sperm surface overlying plasma membrane destroyed (partially or completely)

and is eventually removed and lost from main body of the sperm. After the acrosome reaction the stable and intact inner acrosomal membrane (Barros et al., 1996) is later exposed out. Acrosome reacted sperm swims through the pore of the zona pellucida and fuse with the oolemma (Wassarman, 1995).

Seminal Plasma

The fluid portion of the semen formed after ejaculation is called as seminal plasma. It includes the secretions of accessory glands - ampullae, prostate, vesicular glands and bulbourethral (Cowper's) glands along with secretions of testes and epididymis. The seminal plasma is composed of proteins, amino acids, enzymes, carbohydrates, lipids, minerals, ions, energy substrates, nitrogenous components, reducing substances and organic compounds. The chemical composition and function of seminal plasma fluctuate in different male species and their ejaculates (Reviewed by Nasrin and Stellata, 2012).

Seminal plasma plays important role in ejaculation and motility of sperm and their survival in the female reproductive tract. It acts as a very good buffer providing optimal osmotic medium preventing premature activation with capacitation inhibitors. It defends against phagocytosis. Seminal plasma accelerates ovulation in cows, induces ovulation in pigs and camelids and triggers the expression of embryo trophic cytokines e and organizes the maternal tract for the developing embryo to accommodate gestation. It has influence over fertility, oxygen uptake, motility and sperm quality parameters. Seminal plasma is accountable for the coagulation of semen also. Slightly acidic seminal plasma is seen in bull and rams and slightly alkaline in boar, stallion and camelids. It acts as a carrier and protector of spermatozoa in ewe and cow where ejaculate is deposited in the vagina. (Nasrin and Stellata, 2012).

Seminal plasma proteins

The seminal plasma specific proteins of blood origin are pre-albumin, albumin, globulin, transferrin, α-antitrypsin, β-glycoprotein, β-lipoprotein, peptide hormone, orsomucoid, kininogen, FSH, LH, prolactin, IgG, IgA, IgM (Dondero et al., 1984). They play major roles like regulation of osmotic pressure and pH of seminal plasma, transport of ions, lipids and hormones. The seminal plasma has seven and five protein components in buffalo and cattle respectively. Protein components of low molecular weights are more in buffalo compared to cattle (Ganguly, 1979).

Seminal plasma specific proteins originate from testes, epididymis, and accessory sex glands. Their biosynthesis and secretion is regulated by testosterone level in the blood. The major proteins include Heparin-binding proteins, Non-heparin binding proteins, Bovine Seminal plasma Proteins (BSP family), Bovine sperm forward motility protein (FMP), IgG-Fc binding protein, Cellular retinol binding protein (CeBP), Seminal plasmin, alphalactalbumin, Sperm adhesines, Calsemin, Osteopontins, Immobilins, Inhibin, Clusterin, Androgen binding proteins, Gossact, Interleukins and Insulin Growth Factor system (IGF) (Kulkarni, 2003).

Proteases and protease inhibitors of spermatozoa

The proteases of spermatozoa are contained in the acrosomal vesicle and seminal plasma (Tulsiani et al., 1998). The best known of these proteases are acrosin and hyaluronidase (Yu et al. 2009). Acrosin, a serine protease synthesized during spermatogenesis. It is present in spermatids as an inactive zymogen proacrosin (Parrish et al., 1979) which is converted into the active enzyme during capacitation by a series of endo-proteolytic cleavages of proenzyme from N- and C-terminus region (Baba et al., 1989a and Hardy et al., 1991).It can be stimulated by glysosaminoglycans of the uterine fluid and ovarian follicular fluid in bovine, rabbit, and porcine sperm during capacitation (Reyes et al., 1984).

The proacrosin contains two domains with marked homology to other serine proteases and COOH- terminal tail domain that is unique in the super family (Baba et al., 1989). Domain I, the zymogen domain, contains signal sequences and light chain portion of the active enzyme. During activation of the precursor, the light chain is cleaved from heavy chain arginine and valine at positions 23 and 24 but remains attached to heavy chain by two disulphide linkages. Domain II, the catalytic domain, contains conserved active site residues (histidine, aspartate and serine at positions 70, 124 and 222) and two asparagine linked glycosylation sites. A carbohydrate binding domain in the cat-

alytic domain of boar proacrosin have been identified which play a role in binding of sperm to the ovum. 12 cysteine residues in domain I and II are conserved in all proacrosin and eight of these conserved in serine proteases. Domain III at COOH- terminal end of proacrosin is less conserved between species and is not present in other species. The tail domain is lost during conversion from proacrosin to mature enzyme by successive proteolytic cleavage yielding multiple acrosin with intermediate molecular weight (Baba et al., 1989). Proacrosin is mainly localized to the inner acrosomal membrane in boar, bull and rabbit (Garner et al., 1977).

Acrosin is involved in spermatozoon–egg interactions in the process of fertilization (Palmer et al., 1973) and in the acrosome reaction (Urch and Patel, 1991 and Howes et al., 2001). That time acrosomal content undergo exocytosis and releases the hydrolytic enzymes, digest and pierce the zona pellucida matrix and finally fuses with the oolema (Moreno et al., 2002). Keeping the inactive state of sperm proteases is necessary for maintaining cell integrity for assisted reproduction (Uhrin et al., 2000). From dead/damaged sperm also acrosin is released and appears in the seminal plasma. Acrosin assay can be carried out to test the extent of cryodamage to the sperm (Slowinska et al., 2012).

Hyaluronidase and acrosin are the most important acrosomal enzymes that play an important role in fertilization. Multiple oligomeric forms of hyaluronidase are present in bull and ram spermatozoa (Harrison and Gaunt, 1988). Hyaluronidases in semen are only of sperm origin. It's properties differ from enzymes of lysosomal origin. From dead or damaged sperm, large portion of HA released from acrosome and appears in seminal plasma. It can be used as best 'marker for acrosomal integrity and freezability'. Hyaluronidase helps to penetrate cumulus oophorus of ovum. HA is present in fresh and frozen seminal plasma. Fresh semen has only 10-15% of the total HA in seminal plasma which can withstand cold treatment during freezing in extenders. Hyaluronidase test has been used as a marker for acrosomal integrity and freezability of semen sample for cryopreservation (Ganguly, 1979). Hyaluronidases are glycosidase abundant in acrosome especially in principal segment in bull (Mancini et al., 1964) and ram sperm (Morton et al., 1975). In ram sperm outer acrosomal membrane also have half of the hyaluronidase of the sperm with intact acrosome (Harrison et al., 1988).

Two forms of proacrosin (55 and 53 KDa) and three forms of acrosin, α, β, and γ have been previously identified (49 KDa, 35 KDa and 25 KDa respectively) in ejaculated boar spermatozoa (Polakoski and Parrish, 1977). A trypsin like enzyme (acrosin) has been purified from the acrosome of boar as well as human sperm (Polakoski et al., 1973) and acidic protease with a pH optimum of 2.8 from the acrosomes of bull and ram's sperm. In turkey, acrosin has three subunits of molecular weights below 20,500 (Thurston et al, 1993). A second form of acrosin (acrosin-II) with molecular mass of 30.869 kDa has been identified in turkey spermatozoa (Słowinska and Ciereszko, 2012). During chromato focusing, the acrosin-II was eluted at pH range from 6.4 to 6.2. A neutral protease with a pH optimum of 8.0 has been purified 25-fold from human seminal plasma (Syner and Moghissi, 1972; cited from Ruenwongsa and Chulavatnatol, 1975) which digests proteins in seminal plasma and cervical mucus and may thereby facilitate the migration of sperm in these fluids. In boar, study of epididymal samples showed α- and β-acrosin expression (Puigmule et al., 2011) low in the caput region. But in in vitro capacitated samples, acrosin activity was 2.25 times higher than in the ejaculated samples.

Lytic enzymes including hyaluronidase (Hong et al., 2009), trypsin like enzyme (acrosin), were identified in acrosomal contents. B N-acetyl glucosaminidase is purified from bull, rabbit, human and rhesus monkey spermatozoa occur in close association with hyaluronidase and acrosin on the inner acrosomal membrane, plays an important role in fertilization(Stambaugh and Buckley, 1972). Hyaluronoglucosaminidase is also present in acrosome. Cattle semen has 2.5 times more enzyme level in acrosome than buffalo semen on the basis of enzyme activity in one ejaculate (Kher and Anand, 1974). The western blot analysis of fresh and frozen/thawed spermatozoa showed proacrosin, alpha- and beta-acrosin, with 40-, 32- and 27-kDa bands respectively (De Los Reyes et al., 2009).

Corona penetrating enzyme is non-specific, heat labile enzyme present in the acrosome. Proteolytic enzymes like plasminogen activators 1 and 2 (69 and 74 kDa) have been isolated from human seminal plasma (Propping et al, 1978). They play important role in fertilization. The bovine spermatozoal head is having

a trypsin-like serine protease BSp66 (Cesari et al., 2004a, b). It dimerises when cryopreserved and forms BSp120 (Cesari et al., 2003). There are evidences of aspartate aminotransferase (AAT), hyaluronidase aminotransferase (HAT) and lactic dehydrogenase (Bhosrekar et al., 1994) in spermatozoa of bovine and GOT GTP, AKP, ACP and LDH in buffaloes (Dhami and Kodagali, 1990 and Dhami and Sahni, 1994).

Some other proteases like lysosomal and acrosin forms of β-galactosidase from acrosome and testes of ram and rabbit (Majundar and Lessin, 1975), 110 kDa collagenase with pH optimum of 7.5 from human and bull spermatozoa (Koren et al., 1973), Cathepsin-D proteases from mouse testes (Erickson et al., 1974), and sperm proteases like Dipeptidyl protease-II from guinea pig (Talbot et al., 1985), 80 kDa Calpain- II (Shollmayer,1986) and aryl sulphatases (Dudkiewicz, 1984) from boar, neuraminidases (Srivastava and Abu, 1977) and acid phosphatases from rabbit (Gonzales et al, 1973), non-specific esterase (Brugan et al., 1972) and aryl amidases (Meizel and Cotham, 1972) from bovine, aspartyl amidases from mammalian spermatozoa (Bhalla, 1973) have been reported. Presence of some major proteases was detected in cattle bull semen by Ferrer et al. (2012). Recently Gurupriya et al. (2014) have identified some major and minor proteases in cryopreserved buffalo and cattle semen.

Proteases and proteases inhibitors of seminal plasma

In boars gelatinases (225, 78 and 66 KDa) MMP-9, proMMP-2 and mature MMP-2 are identified in seminal plasma (Pipan et al., 2010). Metayer et al. (2002) identified gelatinases in the ram, boar and stallion. The MMPs are matrix metalloproteinases, degrade the extracellular matrix at physiological pH in a zinc-dependent manner. They have been involved in cell differentiation and connective tissues remodeling (Woessner et al., 1991). Matrix metalloproteinases and their tissue inhibitors play a key role in many physiological processes, including ovulation, fertilization and implantation (Hulboy et al., 1997). Metalloproteases like MMP-2, MMP-9 and serine proteinases have been detected in turkey and human seminal plasma (Kotlowska et al., 2005) and their latent forms in canine seminal plasma and deteriorated semen samples with unsatisfactory quality traits (Tentes et al., 2007). The inactive forms were inversely correlated with semen quality and active forms were positively correlated with semen quality traits and sperm functionality (Saengsoi et al., 2011).

Serine proteolytic enzymes of molecular weights ranging from 29 - 88 kDa and proteinase inhibitor have been identified in turkey (Kotlowska et al., 2005). The ductus deferens (Thurston et al., 1993) and epithelial cells of the epididymal region (Holsberger et al., 2002) are the main site were more proteolytic activity is observed. The metalloproteinases are also present in turkey semen (Metayer et al., 2002; Buchman-Shaked et al., 2002; Shimokawa, 2002). Turkey seminal plasma enzyme (TSPE) (Thurston et al., 1993) of 28-32 kDa and 38-44 kDa having physical, electrophoretic, and kinetic properties distinctly different from those of spermatozoal acrosin of below 20.5 kDa. In chicken seminal plasma no significant proteolytic activity has been detected at basic pH unlike acidic and neutral pH (Droba, 1986).

A 32–34 kDa prostate-specific antigen synthesized in epithelial cells of prostate gland is isolated from human seminal fluid after it has been secreted (Waheed et al., 2008). A 52 kDa protein expressed on the seminal vesicle, seminogelin is known to play an important role in spontaneous coagulation and liquefaction of human semen (Matsuda et al., 1994). PSA causes liquefaction of the semen by cleavage of seminogelin and results in progressive release of motile spermatozoa and is capacitated (Robert et al., 1997).

Generally proteolytic enzymes are active at acidic or neutral pH. Human seminal plasma contains seminin and seminal pepsin, with optimal activities at pH 7.5 and pH 2.5-3.5, respectively (Suominen et al., 1974). Seminin like protease is isolated from seminal fluid of dog, rabbit, and bull (Morton, 1977). Kobayashi et al. (1991) reported basic arginine esterase activity in human seminal plasma and Thurston et al. (1993) detected basic amidase activity in the seminal plasma of the domestic turkey. The amidases activity in turkey was greater than in guinea fowl or chicken and that too in vas deferens than testicular or epididymal fluids.

Dilution of semen has been shown to disturb equilibrium between sperm coating proteins and those in solution (Pavelko and Crabo, 1976). Acrosome and

seminal plasma are rich in acrosin inhibitors (Janakova et al., 1991). Acrosin activity is inhibited by Kazal type inhibitors present in seminal plasma (Slowinska et al., 2008) that binds to acrosin during ejaculation and are removed during capacitation (Zaneveld and Williams, 1970; cited from Gilboa et al., 1973). The sperm proteases must be in an inactive form for keeping cell integrity and maintaining the sperm function (Zheng et al., 1994). Here comes the protective role of proteinase inhibitors by inhibiting the proteolytic action of acrosin liberated from the acrosomes of dead and damaged spermatozoa and maintains structure of sperm duct epithelial cells, seminal plasma proteins, or viable spermatozoa (Suominen et al., 1972). Proteases and pronases were inhibited relatively more by buffalo and cattle seminal plasma respectively (Kakar and Ganguly, 1978).

Serine proteinase inhibitors have been detected in turkey testis, epididymis, ductus deferens, spermatozoa surface and in seminal plasma (Kotlowska et al., 2005) using electrophoretic methods. They have Kazal family inhibitor in their seminal plasma inhibiting trypsin-like enzymes in vivo (Slowinska et al., 2008) and hence known as acrosin inhibitors (Laskowski and Kato, 1980). Chicken seminal plasma contains inhibitors of spermatozoa acrosin, a serine protease with optimum activity between pH 8 and 9 (Morton, 1977).

In human and chicken seminal plasma species specific acrosin-trypsin inhibitors, (Fink et al., 1990 and Lessley et al., 1978), in bull seminal plasma and washed ejaculated spermatozoa Inhibitor I of 8.7kDa & Inhibitor II 6.8kDa (Cechova and Fritz, 1976) and in boar seminal vesicle fluid 9.5-12 kDa inhibitor have been identified (Veselsky et al., 1985). Anti-proteinase activity that inhibiting bovine or cod trypsin of 90 kDa was identified in seminal plasma of teleost fish. They have serpin like activity. Gurupriya et al. (2014) demonstrated some major and minor proteases in buffalo and cattle seminal plasma with majority having metalloproteinase activity.

CONCLUSION

Semen is a liquid cellular suspension comprising spermatozoa, the male gamete and accessory gland secretion. It is enriched with various proteases and their inhibitors. Exact functions of these enzymes are currently unknown. The assessment of these enzymes and their role in sperm functions should be taken into account in assisted reproduction. The status of these proteases and their inhibitors before and after cryopreservation, liquid storage provide a powerful stimulus for extending the improvement of semen preservation protocols and development of better diluent preparation.

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