# Proteomics of boar seminal plasma

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Proteins are the major catalysis of biological function and contain several dimensions of information that collectively indicate the actual and the potential functional state of a cell or a tissue of an organism. Hence, proteomics holds a key position in the new biology.

The major goal of proteomics as sciences is making inventory of all proteins encoded in the genome.

(PROTE**in** complement to a **gen**OME) of a certain organism and analysis of interaction of these proteins (Carbonaro, 2004).

Several subcategories of proteome research have been developed in the past couple of years.

These include: “expression proteomics”, which has the primary goal of mapping out a particular proteome or subproteome; “quantitative proteomics”, which aims to compare the relative abundance of a particular proteome under defined conditions; “functional proteomics”, with the goal of defining the complete network of cellular protein-protein interactions; “structural proteomics”, which is the science of exploring the three – dimensional structure of every cellular protein; and “posttranslational modification proteomics”, which aims to map the exact number and position of various posttranslational modifications across the proteome.

Presented strategy used to analyze the mammalian proteome has shown that proteomics consists not only of the identification and quantification of proteins, but also is the study of their structure, localization, modification, interactions, activities and functions (Wasinger, Corthals, 2002).

Methodologically, proteomics is based on highly efficient methods of separation and analysis of proteins in living systems.

Classically, two-dimensional (2-D) gel electrophoresis performed as a combination of isoelectric focusing and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) had been the only method to analyze the proteome with high resolution. With this method, the thousands of proteins that are expressed in a specific cell or a specific tissue can be separated (Görg, 2000).

Hitherto, no method had been developed with a resolution greater than 2-D gel electrophoresis. The differentially expressed proteins in response to changes in cellular states are especially focused and analyzed with this method. This methodology, termed "expression proteomics" worked as a major driving force behind proteomics analysis. However, the conventional 2-D electrophoresis only shows protein expression and cannot detect protein-protein interactions and protein functions in principle without using particular methods such as affinity chromatography or gel chromatography (multi – dimensional chromatography). Thus, other approaches are required to enable a comprehensive understanding of cellular mechanisms at the protein level. For this purpose, we need to now how each protein acts and which proteins are functionally interrelated.

This emerging field of systematic protein analyses focusing on protein function, its interactions and biological phenomena is termed "functional proteomic" (Yanagida, 2002).

This presentation concerns a survey of some recent developments in proteomics of boar seminal plasma and its possibility to use as a new marker of semen biological value.

The multifunction of seminal plasma proteins is facilitated by their structure and action of molecular mechanism. These proteins are a new tool in the control of molecular mechanism accompanying sperm transport in the female reproductive, suppression of the female immune response against sperm antigens, and gamete interaction following egg fertilization. However, the development of the *in vitro* fertilization techniques and embryo micromanipulation needs to answer many questions regarding the function of seminal plasma proteins in the fertilization process. For example, pre-incubation of flow sorted boar spermatozoa with seminal plasma increases their ability to penetrate IVM-oocytes (Parilla et al. 2004). Moreover, the regulatory action of seminal plasma proteins is manifested at the level of molecular phenomena accompanying various stages of animal reproduction. Additionally, the role of seminal plasma proteins in semen preservation technology appears to be important (Caballero et al. 2004).

Therefore, it is necessary to analyze the structure and concentration of these proteins with new biochemical methods.

Boar seminal plasma is a highly diluted form of seminal vesicle secretion; diluted to the extent that most polypeptides from other glands and the epididymis are not in sufficient concentrations to be noted, even with the highly sensitive silver-staining technique. Lavon and Boursnell (1971); Lavon et al. (1972) utilizing isoelectric focusing on polyacrylamide gel, estimated that 80-90 % of the protein in seminal plasma was derived from seminal vesicles with the remaining detectable protein from the epididymal fluid (2-5%), prostate (5%) and bulbourethral gland (10-15%).

## 1. Identification of total proteins and differentially expressed proteins of boar reproductive tract fluids – correlations with some biological values of semen

Intensive processed of protein secretion in the seminal plasma following shortly after the boars reached sexual maturity. Evidence has been shown that seasonal variations affect protein secretion in the seminal plasma, irrespective of the boar age (Strzezek et al. 2002).

It is interesting to note that trichloroacetic acid precipitated fractions, containing both of low- and high-molecular weight protein structure, were dominant in electrophoretic profile (PAGE) of boar seminal plasma proteins. Separation of proteins by affinity chromatography with lectin concanavalin A (Con A) showed that a series of polymannose glycoproteins was predominant in boar seminal plasma. The concentrations of these proteins were stabilized during the earlier period of sexual maturity and until the boar were about 2.5 years old. In boar aged three years, these protein concentrations were significantly reduced, indicating that changes in glycosylation process of seminal plasma proteins, synthesized in the reproductive tract, might depend on the boar age.

Disturbances in the quantitative relation of the low- and high-molecular weight fractions, following a detailed analysis of the electrophoretic profile of the seminal plasma glycoprotein fractions. Variations between boars were dependent on the animal age. Even though the molecular mechanism in not yet known, it seems to suggest that changes in the secretory activity of different organs of the reproductive systems are age-dependent. This phenomenon may affect the boar fertility.

Recent evidence has shown that the secretory epithelium of the boar vesicular glands is diverse and involved in a complex glycoconjugate secretory pattern. The glycoconjugates consist mainly of N – and O – glycoproteins, which are implicated in various fertilization-related events (Badia et al. 2005).

The two-dimensional (2-D) gel electrophoresis was utilized for the first time to study proteome of boar reproductive tract by Russel et al (1984). The seminal plasma showed a few major polypeptides from the cauda epididymal fluid, but the major constituents were the vesicular polypeptides, high-molecular polypeptides from either the bulbourethral gland or prostate gland, and another major acidic polypeptide of high-molecular weight from the vesicular glands. Numerous neutral and basic low-molecular weight polypeptides, originating from the vesicular glands, adhered tightly to the spermatozoa. It is interesting that the fluid of a boar seminal vesicle shows about 150 acidic and neutral – range polypeptides, which migrate towards neutrality or in the highly basic region during 2-D electrophoresis.

The question is whether the protein composition of seminal plasma is different for boars of differing fertility.

More recently, Killian et al. (1993) have shown that glycoproteins in seminal plasma differed among bulls varying from high to low non – return rates (two proteins: 26 kDa, 6.2 pI; 55 kDa, 4.5 pI predominated in higher – fertility bulls, whereas two proteins: 16 kDa, 4.1 pI; 16 kDa, 6.7 pI were more prevalent in below average fertility bulls). The authors showed that they could predict the non – return rate of AI bulls from a regression equation based upon seminal content of four specific "fertility – associated" glycoproteins (r = 0.89).

Two – dimensional gel electrophoresis was also used to detect the relationship between seminal plasma proteins and fertility in boars. Flowers (1998, 2000) reported about the biological importance of proteins (26 kDa, pI 6.2 and 55 kDa, pI 4.8) present in boar seminal plasma and claimed that high concentrations of both (relative units greater than 10) in boar ejaculate corresponded with high farrowing rates (over 86%) and number of piglets born alive (over 11). The author reaffirmed the individual variation among boars, as regards the fertility – associated proteins. Laboratory studies have indicated that a minimal amount of seminal plasma is necessary in a dose of semen to sustain the fertility of the spermatozoa after deposition into the female and that the seminal plasma in an AI dose is important in controlling uterine inflammation. The inclusion of 10-12% of seminal plasma to AI dose may have a beneficial effect on the biological value of the semen It appears that, besides the regulatory functions in the fertilization process, seminal plasma proteins play an important role in the suppression of uterine inflammatory response caused by rapid leucocytosis following deposition of boar semen in the sow reproductive tract (Flowers, 2001; Rozeboom 2000).

This study shows that the use of appropriate methods of measuring protein concentrations as well as identification of proteins components in the seminal plasma of individual boars may have important impact on practical evaluation of the biological properties of the semen.

It is interesting to note that the proportion of piglets sired by the dominant boar was lower when the spermatozoa were mixed with seminal plasma from a non – dominant male, whereas the converse was true with the reciprocal combination, non – dominant semen with dominant seminal plasma (Flowers, 1997).

In our recent study, the use of 2-D has shown that there were qualitative changes in the profile of seminal plasma proteins, which were dependent on relation to boar age (Kordan et al 2004). These changes were manifested in the appearance of additional polypeptide fractions with molecular weights of 30-50 kDa and with different pI. It was found that there were 4 conserved proteins, with molecular weights of 30-40 kDa, in the electrophoretic profiles of the seminal plasma of each boar. These proteins were also present in the seminal plasma of European wild boar – domestic pig hybrids. However, the physiological significance of these proteins in the reproductive processes requires further research.

## 2. Functional proteomics of boar seminal plasma – biochemical approaches

This study is based on some selected elements of the multifunctional components of boar seminal plasma, particularly those related to the basic physiological function of spermatozoa in the reproductive processes of swine.

Recent studies have shown that the process of recognition and sperm – egg binding is mediated by the type of carbohydrate protein interactions. The zona pellucida of mammals is composed of only 2 – 4 glycoproteins families, which appear to facilitate its defined biological functions (Töpfer-Petersen et al. 1995, 1999).

The group of sperm coating proteins, which bind to the zona pellucida, are different in relation to their biochemical structure and molecular weights (14 to 90 kDa) and may vary from species to species.

In the last 10 years, scientific workers from Germany (Prof. E. Töpfer-Petersen and team) and Spain (Prof. Juan Calvete and team) had made a series of unique contributions to research based on the protein system of boar seminal plasma. These contributions have been of great interest in molecular biology of fertility process in mammals, particularly in swine.

Generally, seminal plasma contains specific proteins factors that influence both the fertilizing ability of spermatozoa and exert important effects on the female reproductive physiology.

In the boar, the bulk of seminal plasma proteins belong to the spermadhesin family. Spermadhesins are synthesized mainly by the vesicular glands, in some cases by the epididymis and fluid of the rete testis (Calvete et al. 1996).

There are abundant literature regarding the structure and biochemical properties, and physiological functions of boar spermadhesins. Five spermadhesins have been identified in the boar seminal plasma: AQN – 1, PSP – I (AQN – 2), PSP – II, AQN – 3 and AWN (isoforms 1 and 2). The basic structure of spermadhesins in the boar seminal plasma has been reported. Post – translational modification is related to glycosylation that facilitates the differentiation in the functional properties of spermadhesin.

It is interesting to note that glycosylation not only contributes to the structural diversity of the proteins family, but also affects the ligand – binding capabilities of the glycosylated spermadhesins i. e. it abolishes their zona pellucida – binding activity without impairing heparin binding (Calvete et al. 1993, 1994).

AQN – 1, AQN – 2, AQN – 3 and AWN are heparin – binding spermadhesins. Moreover, PSP – II is a major component of the non – heparin binding fraction of boar seminal plasma (Calvete et al. 1995).

Recently, seminal plasma glycoproteins I (PSP – I) and II (PSP – II), which are major components of boar seminal plasma and which form non-covalent heterodimers (Kwok et al. 1993; Calvete et al. 1995) have been of particular interest. It is surprising that PSP – I / PSP – II complex does not bind to the sperm surface, excluding its role in gamete interaction. Although the biological function of PSP-I/PSP-II remains unclear, purified samples of these non – heparin binding spermadhesins have been added to extended boar semen at different andrology laboratories (Centurion et al. 2003). For example, there was an increased in viability of highly diluted boar spermatozoa, evaluated with SYBR-14/PI, when exposed *in vitro* to the glycoprotein subunit of PSP – II (Garcia et al. 2004). Furthermore, the supplementation of the spermadhesin PSP – II / PSP – II complex to a freezing extender did not affect post – thaw sperm survival (Cremades et al. 2004). However, these spermadhesins inhibit *in vitro* fertilizing ability of frozen – thawed boar spermatozoa (Caballero et al. 2004).

Our preliminary studies show that dialysis of boar semen prior to cryopreservation has a beneficial effect on post – thaw sperm motility, plasma membrane integrity, DNA stability DNA and acrosome membrane integrity (Fraser and Strzezek, unpublished).

Recently, it has been confirmed that PSP-I/PSP-II heterodimer stimulates macrophages to release a neutrophil chemotactic substance (Assremy et al. 2003). These data support a role of spermadhesins – heterodimer as a modulator of the uterine immune activity.

To recapitulate, the results of these studies emphasize that some boar seminal plasma proteins may function as structural and sperm – modulating proteins or transport – and immuno-modulating proteins.

Our team has undertaken complex studies on the system of boar seminal plasma protein, particularly those secreted by the seminal vesicle glands (Tab. 1).

## Tab. 1. Protein and peptide substances isolated from boar seminal plasma – by the research team of the Department of Animal Biochemistry and Biotechnology

| Substance | Secretion | References |
|---|---|---|
| $Zn^{2+}$ - ion dependent protein | seminal vesicle | Strzezek & Hopfer [1987]<br>Strzezek et al. [1987] |
| Sperm motility inhibiting factor (SMIF) | seminal vesicle | Kordan et al. [1998]<br>Strzezek et al. [1992]<br>Velev et al. [1992] |
| High – molecular | | Strzezek & Torska [2001] |

| | | |
|---|---|---|
| proteinase inhibitor | seminal vesicle, epididymis | Torska & Strzezek [1995] |
| 54 kDa glycoprotein | seminal vesicle | Holody et al. [1996]<br>Holody & Strzezek (1999)<br>Kordan et al. [1999]<br>Plucienniczak et al. [1999]<br>Strzezek & Holody [1996] |
| Phosphotyrosine acid phosphatase | seminal vesicle | Wysocki & Strzezek [2000]<br>Wysocki & Strzezek [2003] |
| Platelet – activating factor acetylhydrolase (PAF-AH) | seminal vesicle prostate | Kordan et al. [2000]<br>Kordan & Strzezek [2002]<br>Kordan et al. [2003] |
| Superoxide dismutase (SOD) | seminal vesicle | Kuklinska & Strzezek [2004] |

The biochemical and biological properties of the peptide and protein substances isolated in the course of our studies will be presented in more detail in the author's lecture.

It is necessary to underline that we have been ably to purity a unique protein from boar seminal plasma or seminal vesicle fluid, using modern affinity chromatography, mainly columns bound with zinc ions.

The seminal plasma has an important role in maintaining the optimal level of $Zn^{2+}$ ions. Complex studies on the decondensation process of human spermatozoa have shown that the vesicular glands secrete high-molecular weights zinc ligands that reduce zinc content in the sperm chromatin (Kjellberg 1993). On the other hand, the prostatic fluid is rich in free $Zn^{2+}$ ions or $Zn^{2+}$ ions bound with low-molecular weight components, which may act as a modulator of sperm chromatin condensation. Furthermore, disturbances in the Zn/fructose molar ratio may lead to a reduction of zinc levels in the chromatin, and consequently destabilise the sperm chromatin. This phenomenon is attributed to the dominant role of zinc ligands in the vesicular secretion and may be attenuated by the hypo-function of the prostate. Hence, it can be suggested that the determination of the Zn/fructose molar ratio may be used as a diagnostic marker of changes in the chromatin status of infertile human spermatozoa.

The use of radioisotope techniques in our laboratory to evaluate the stability of sperm chromatin has confirmed that intensive sexual exploitation, cryoproterone acetate- antiandrogenesis and atropine administration caused disturbances in the secretion of low- and high-molecular weight components of the seminal plasma. These disturbances had a serious impact on the sperm chromatin status, resulting in the formation of hypo- or hiper-stabilization state. The basic modulator of the sperm chromatin appears to be $Zn^{2+}$ ions and their ligands, originating in the vesicular glands of the boar (Strzezek et al. 1998a, 1998b, 2000).

Accumulating evidence has shown that the seminal plasma plays a pivotal role in the regulation of sperm motility. Recent research on peptide inhibitors of sperm motility, which have been isolated from human and boar seminal plasma, has been remarkable. Some authors have presented extensive information on the seminal plasma inhibitors of motility of boar spermatozoa (Kordan et al. 1998). These peptide substances, originating in the vesicular secretions, have similar structural homology or/are important spermadhesins of AQN-3 and DQH of boar seminal plasma. This may suggest a role of plasma membrane receptors at the region of the middle and tail pieces in the regulation of mammalian sperm motility apparatus.

Boar spermatozoa are exceptionally susceptible to the action of reaction oxygen species (ROS) because they have a high concentration of polyunsaturated fatty acids in their plasmalemma and inadequate enzyme antioxidant system in their cytoplasm of the middle piece. Besides spermatozoa, leucocytes may also contribute to the source of ROS generation. ROS generation is related to the sperm physiological function and is enhanced during deterioration of the sperm morphological structures.

Sperm capacitation and acrosome reaction are mainly stimulated by radical superoxide, which production is related to the activity of membrane-bound-oxidase NADH, regulated by $Ca^{2+}$ ions and protein kinase C. Disturbance in the equilibrium between pro-oxidant and antioxidant systems of semen, may lead to increased ROS generation, which may affect sperm motility, survivability and chromatin status. The antioxidant properties are facilitated by the occurrence of low- and high-molecular weight components of the seminal plasma. Besides the antioxidant enzymes, thermostabile dismutase superoxide and glutathione peroxidase, there are non-specific low-molecular weight substances, such as reduced L-glutathione, L-ergothioneine, L-ascorbic acid, tocopherol and uric acid. It should be noted that the concentrations of these substances are species-dependent.

Seminal plasma proteins play a significant role in the defence of spermatozoa against ROS. Vesicular $Zn^{2+}$-dependent protein of boar seminal plasma has been shown to possess antiperoxidant properties (Strzezek et al. 1999). However, boar spermatozoa isolated from seminal plasma seem to have a very weak enzymatic system against hydrogen peroxide and organic peroxide.

Our electrophoretic studies have revealed three molecular forms of superoxide dismutase (SOD) and one form catalase in stallion spermatozoa, whereas only one molecular form of SOD and a lack of catalase activity was revealed in boar spermatozoa. Stallion spermatozoa also showed glutathione peroxidase (GPx) activity (Strzezek et al. 2000).

The low levels of SOD and the lack of GPx and catalase in the seminal plasma indicate that boar spermatozoa are poorly adapted to counteract the toxic effects induced ROS. This has been compensated by the pivotal role of the sulphur – containing antioxidants (L-glutathione and L-ergothioneine) and L-ascorbic acid as well as the high protein antiperoxidant activity of boar seminal plasma.

The relationship between the total protein content and antioxidant defence system in boar seminal plasma can be used to create new possibilities for diagnosis regarding the molecular aetiology of subfertility and infertility in the boar.

It is noteworthy that the level of $Zn^{2+}$ ions of the seminal plasma is highly correlated with the activity of trypsin inhibitors.

Besides the antiperoxidant properties of seminal plasma, immuno-modulation protein substances play an important role in the suppression of formation of antisperm antibodies. For example, in the boar our previous studies showed that the heterogeneous 54kDa glycoprotein of seminal plasma and vesicular fluid blocked spontaneous proliferation of lymphocytes. It is interesting to note that one fraction of 54kDa glycoprotein, with a molecular weight of 15kDa, displays binding capability towards pig IgG (Strzezek, Holody, 1996). Similar observations have been confirmed for human seminal plasma.

The physiological significance of the presented properties of seminal plasma proteins in relation to their binding to IgG is not fully clear. However, it can be suggested that this phenomenon is related to the sperm defence against humoral and cellular attack mediated by the autoimmumological system. The discussed mechanism is probably synchronized with limited immunological reactivity of the female against sperm antigen during the process of egg fertilization.

Seminal plasma is rich in antibacterial substances, such as zinc and the chelating action of protein, lysozyme, seminalplasmine, spermine and glycosidase.

Further improvement in the biotechnological methods in reproduction of swine needs to elucidate the action of seminal plasma components in the reproductive process. Moreover, many factors should be considered as regards the functional biochemistry of components of boar seminal plasma.

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