

Application of mass spectrometry (MS) in the structural analysis of selected polypeptides of boar seminal plasma obtained after two-dimensional electrophoresis (2-D PAGE)*

Władysław Kordan^{1,}, Agata Malinowska²,
Marzena Mogielnicka¹, Marek Lecewicz¹**

¹ Department of Animal Biochemistry and Biotechnology,
University of Warmia and Mazury in Olsztyn,
10-917 Olsztyn-Kortowo, Poland

² Polish Academy of Sciences Institute of Biochemistry and Biophysics,
Pawieńskiego 5a, 02-106 Warsaw, Poland

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Two-dimensional electrophoresis (2-D PAGE) led to identification in the polypeptide maps of boar seminal plasma of four conserved polypeptides with identical molecular weight of 24 kDa, and different ranges of isoelectric point (pI): (1) 7.4-7.7, (2) 8.1-8.4, (3) 8.5-8.8 and (4) 9.2-9.4. In the current study the molecular structures of these polypeptides were analysed, for the first time, by mass spectrometry (LC – MS/MS). Computerized mass spectrometry analysis of the peptides obtained after trypsin-digestion of the polypeptides demonstrated their similarity to the family of spermadhesins (crystal structure of two members of the spermadhesin family), especially to epididymal spermadhesin AWN-1. In addition, homology was found of peptides 3 and 4 with a lysozyme C precursor (1,4-beta-N-acetylmuramidase C). The results presented might indicate the participation of the analysed polypeptides in the processes accompanying fertilization.

KEY WORDS: boar / 2-D PAGE / mass spectrometry / proteins
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**Corresponding author: wladyslaw.kordan@uwm.edu.pl

Seminal plasma of mammals is a mixture of secretions originating from the testes, epididymes, accessory sex glands and ejaculatory ducts. Components of seminal plasma, in particular proteinous and peptide substances, play an important role in the function regulation of the male gamete. Seminal plasma contains a variety of components, including regulators of capacitation and acrosome reaction, fertility proteins, enzymatic proteins, proteins influencing semen freezability and regulators of motility of sperms [Shivaji *et al.* 1990]. These substances, secreted at ejaculation, are associated with major functions of spermatozoa, that is, with their motility and fertilizing ability.

In mammals, fertilization involves a number of specific interactions between substances bearing traits of ligands and receptors that occur at the surface of the reproductive cells. It should be mentioned that, after ejaculation, most of the seminal plasma proteins bind to the surface of the plasmalemma of spermatozoa, thus determining their fertilizing ability. Moreover, some of these substances could disassociate from the plasmalemma of spermatozoa during their migration in the female reproductive tract [Strzeżek *et al.* 2005]. During migration, spermatozoa undergo specific changes associated with their maturation process, referred to as capacitation [Breitbart 2002]. Capacitation is aimed at triggering structural changes in the plasmalemma of a spermatozoon, resulting in the exposure its receptors for binding with the *zona pellucida* to enable the initiation of the acrosome reaction prior to sperm-egg fusion. In the regulation of these processes a significant role is ascribed to low-molecular proteins – spermadhesins, which are mainly located at the apical part of the acrosome region of the sperm head. These substances are thought to be capable of the recognition of receptor sites and binding of spermatozoa with receptors of the *zona pellucida* [Haase *et al.* 2005].

Analysis of the biochemical characteristics of seminal plasma proteins requires the application of modern analytical methods. Recently, the two-dimensional electrophoresis (2-D PAGE) has been successfully applied for mapping proteins of boar seminal plasma [Kordan *et al.* 2004, 2008]. The method has enabled the identification of individual polypeptides in the complex protein structure. An investigation presented here has demonstrated, among others, quantitative and qualitative changes in the composition of the polypeptide maps of the seminal plasma, as related to season and boar age. Nevertheless, in all the analysed samples of the seminal plasma, we have identified four conserved polypeptides, each having a molecular weight of 24 kDa and different isoelectric point, ranging from 7.4 to 9.4. This study aimed at analysing the structural traits of the above-mentioned polypeptides of boar seminal plasma, using mass spectrometry.

Material and methods

Obtaining the seminal plasma

Ejaculates were collected manually from three boars of the Polish Large White and Polish Landrace breeds kept at the Laboratory of the Biology of Reproduction, Department of Animal Biochemistry and Biotechnology, University of Warmia and Mazury in Olsztyn. Analyses were conducted on 16 ejaculates. The study was approved by the Local Ethics Committee, permission no. 42/2007.

After collection, the ejaculates were filtered through sterile gauze in order to remove the gelatinous fraction and centrifuged twice, first at 900 x g for 20 min and then at 10,000 x g for 20 min at room temperature. The resulting seminal plasma was kept at -80°C (193.2 K), until electrophoretic separation.

Preparation of seminal plasma samples for electrophoretic separation with 2-D PAGE

Prior to electrophoretic separation, protein content was determined in the samples according to Lowry *et al.* [1951]. The samples were diluted to a final concentration of 0.1 mg protein/ml and pre-incubated for purification with a Plus One 2-D Clean-Up reagent Kit, (AMERSHAM BIOSCIENCES) according to the producer's recommendations. After pre-incubation, the samples were suspended in 100 µl of lysis buffer (9.5 M urea – AMERSHAM BIOSCIENCES, 2% Triton X-100 – SIGMA, 65 mM (0.65 x 10⁻¹ M) DTT – SERVA, 2% solution of ampholytes in pH range of 3 to 10 – AMERSHAM BIOSCIENCES, 0.1% Bromophenol Blue - SIGMA) and stored at -80°C (193.2 K), until 2-D PAGE analysis.

2-D PAGE of seminal plasma proteins

The 2-D PAGE was conducted according to O'Farrel *et al.* [1977], in a pH gradient of 3 to 10 and 6 to 10, with modifications.

Isoelectrofocusing (IEF)

A purified sample of seminal plasma proteins was diluted in a lysis buffer as described above, to reach a final concentration of 75 µg/145 µl. The purified sample was applied onto a strip (ZOOM Strip, pH 3-10 NL or 6-10 NL, INVITROGEN) fixed in a cassette (ZOOM IPG Runner cassettes, INVITROGEN). The cassette was mounted in an apparatus (ZOOM IPG Runner, INVITROGEN) and the electrophoretic separation was run in deionized water using variable voltage (20 min – 200 V, 15 min – 400 V, 15 min – 700 V, 120 min – 1,000 V). On completion of the isoelectrofocusing, the strips were stored at -80°C (193.2 K), until analysed.

Denaturing electrophoresis (NuPAGE)

After isoelectrofocusing, the strips were equilibrated for 30 min in a buffer of the following composition: 6 M urea, 2% SDS (SIGMA), 0.375 M Tris, 20% glycerol (POLISH CHEMICALS Inc., Poland), 2% DTT and 0.2% Bromophenol Blue, and for

10 min in an identical buffer containing of 2.5% iodoacetamide (SIGMA). Afterwards, the strips were placed onto a previously prepared 10% electrophoretic polyacrylamide NuPAGE (INVITROGEN) gel. Electrophoretic separations were conducted in a buffer of the following composition: 250 mM MES (2-N morpholino ethane sulfonic acid), 250 mM Tris, 5 mM EDTA (POLISH CHEMICALS Inc., Poland), 0.5% SDS (SIGMA); 24.5 mM sodium bisulfite (SIGMA), using a Mini Protean II apparatus (Bio-Rad) and different voltages (15 min – 80 V and 50 min – 130 V).

Detection of samples after 2-D PAGE electrophoresis

Polyacrylamide gels were stained with the silver method [Shevchenko *et al.* 1996]. Electrophoregrams were analysed with PD Quest™ 6.2 software (Bio-Rad). After the analysis of electrophoregrams, individual polypeptides were cut out from the gels and stored in 1% acetic acid until further analyses.

Mass spectrometry (MS)

The spectrometric analysis of the polypeptides obtained was carried out in the Environmental Laboratory of Mass Spectrometry, Institute of Biochemistry and Biophysics of the Polish Academy of Sciences in Warsaw. The samples were analysed with liquid chromatography coupled with tandem mass spectrometry (liquid chromatography/two stage mass spectrometry – LC-MS/MS), according to the procedure described by Kordan *et al.* [2007]. The output list of precursor and product ions was compared with the protein database of the National Center for Biotechnology (NCBI), USA using the MASCOT local server.

Results and discussion

The use of 2-D PAGE at a pH gradient of 3 to 10 enabled to obtain polypeptide maps of boar seminal plasma proteins. Interestingly, analyses of their composition demonstrated the presence of four conserved polypeptides, with identical molecular weight of 24 kDa, but different isoelectric point: (1) pI 7.4-7.7; (2) pI 8.1-8.4; (3) pI 8.5-8.8; and (4) pI 9.2-9.4, respectively (Fig. 1). Taking into account the obtained pI values of these polypeptides, electrophoretic separations of the seminal plasma proteins were conducted at a pH gradient of 6 to 10 (Fig. 2), giving a higher resolution of electrophoregrams and, consequently, enabling easier isolation of these substances for mass spectrometry analysis, as shown in Table 1. It should be mentioned that recent investigations [Kordan *et al.* 2004] have demonstrated the presence of these substances in all analysed seminal plasma samples obtained from boars aged 6 to 42 months.

In this study, the use of computerized mass spectrometry showed, for the first time, that the analysis of the peptides obtained after trypsin-digestion of the polypeptides demonstrates their similarity to the family of spermadhesins (crystal structure of two members of the spermadhesin family), especially to epididymal spermadhesin

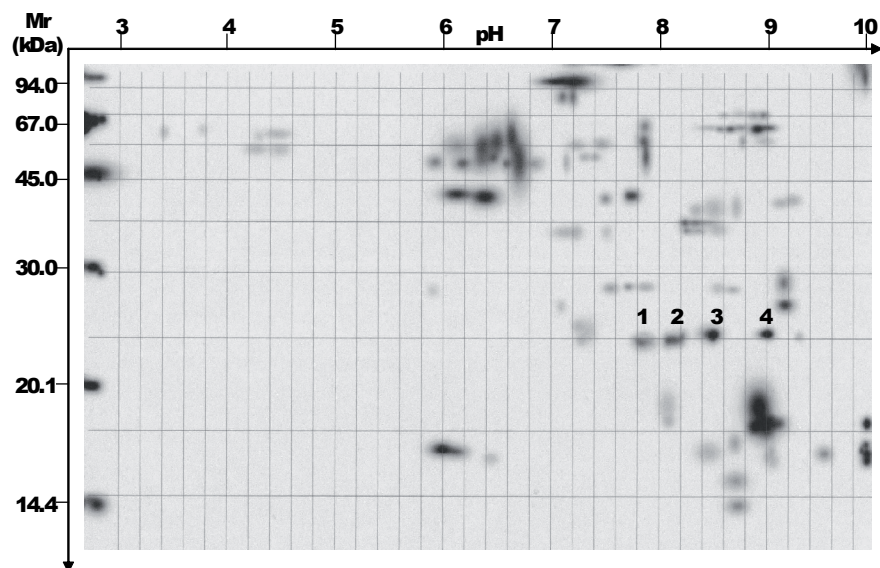


Fig 1. Two-dimensional electrophoresis (2D-PAGE) of boar seminal plasma in a pH gradient 3-10. 1, 2, 3, 4 – polypeptides.

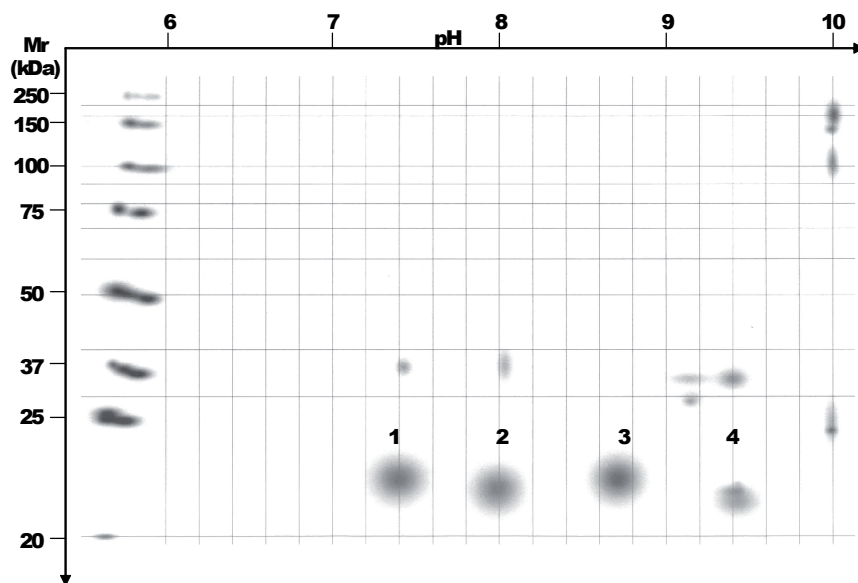


Fig 2. Two-dimensional electrophoresis (2D-PAGE) of boar seminal plasma in a pH gradient 6-10. 1, 2, 3, 4 – polypeptides.

Table 1. Analysis of the conserved polypeptides from boar seminal plasma using mass spectrometry

Poly-peptide (no.)	Protein	Molecular weight of polypeptide (Da)	Homologous amino acid sequence
1	Crystal structures of two members of the spermadhesin family	1151 1349	DTSGSISNTDR MAIPYLNACGK
	AWN-1 spermadhesin	919	SSSNIATIK
2	Crystal structures of two members of the spermadhesin family	1151	DTSGSISNTDR
		1151	DTSGSISNTDR
		1858	EYVEVFDGLLSGPSYGK
		1858	EYVEVFDGLLSGPSYGK
3	Crystal structures of two members of the spermadhesin family	919	SSSNIATIK
		919	SSSNIATIK
	Lysozyme C precursor (1,4-beta-N-acetylmuramidase C)	1151	DTSGSISNTDR
		1858	EYVEVFDGLLSGPSYGK
		919	SSSNIATIK
		919	SSSNIATIK
4	Crystal structures of two members of the spermadhesin family	1151	DTSGSISNTDR
		1365	MAIPYLNACGK
		1407	DTSGSISNTDRQK
		1491	VIKDTSGSISNTDR
	AWN-1 spermadhesin	1858	EYVEVFDGLLSGPSYGK
		919	SSNIATIK
		919	SSNIATIK
		1427	FESNFNTQATNR
	Lysozyme C precursor (1,4-beta-N-acetylmuramidase C)	1427	FESNFNTQATNR
		1752	NTDGSTDYGILQINSR

AWN-1. In addition, in the case of polypeptides 3 and 4, analyses demonstrated their homology to precursor of lysozyme C (1,4-beta-N-acetylmuramidase C).

Spermadhesins are a group of low-molecular proteins of the seminal plasma (12-16 kDa), which display strong adhesive properties and lectin-like activity, and are implicated in sperm-egg interaction [Haase *et al.* 2005]. These substances also exhibit ligand-binding ability to heparin, serine proteinase inhibitors, phospholipids and carbohydrates [Calvete *et al.* 1995a]. Two fibronectin domains have been identified in the structure of spermadhesins. Fibronectin is a glycoprotein with a dimeric structure that displays adhesive properties and strong affinity for glycosaminoglycans and gangliosides, and binds receptors of various cells [Manjunath and Thérien 2002, Strzeżek 2002]. The presented properties of fibronectin increase the sperm binding ability to receptors of the *zona pellucida* [Calvete *et al.* 1995a]. Spermadhesins also function as regulators of capacitation and acrosome reaction in spermatozoa and act as immunosuppressive factors during the transport of spermatozoa in the female reproductive tract [Kwok *et al.* 1993].

Presently, 6 spermadhesins have been identified in boar seminal plasma: AQN-1, AQN-2 (PSP-I), AQN-3, AWN, DQH and PSP-II [Calvete *et al.* 1994, 1995ab, Plucienniczak *et al.* 1999]. These substances display 40-60 % amino acid sequence similarity. The AWN spermadhesin possesses two isoforms (AWN-1 and AWN-2) that differ due to the presence of an acetylated alanine in the N-terminal sequence of AWN-2. It should be noted that AQN-2 (PSP-I) and PSP-II are glycoproteins, whereas AQN-3 and AWN (isoforms 1 and 2) occur in the non- and N- and O-glycosylated forms. Presently, no new carbohydrate components have been identified in the structure of the AQN-1 protein. AQN protein group occurs mainly in the seminal vesicles [Calvete *et al.* 1994, 1995b], whereas AWN proteins are secreted by the *rete testis* and *tubuli recti* [Calvete *et al.* 1994, Dostálová *et al.* 1995]. Noteworthy is the detection of the AWN spermadhesin on spermatozoa bound to the *zona pellucida* in vivo [Rodríguez-Martínez *et al.* 1998]. This phenomenon indicates that the AWN spermadhesin may modulate the fertilizing ability of epididymal spermatozoa. Recent studies have demonstrated that porcine AWN spermadhesin is also secreted in the female reproductive tract, especially in the Fallopian tube [Ekhlas-Hundrieser *et al.* 2002]. The DQH spermadhesin is a protein comprising of a 25-amino acid signal peptide. This substance was obtained by cloning the complementary structure of DNA. Homology has been shown of the amino acid sequence of DQH with that of the sequence of spermadhesins from bull (BSP-A1/A2 and BSP-A3) and stallion seminal plasma (HSP-1) – Plucienniczak *et al.* 1999]. Moreover, DQH has also been demonstrated to reduce the percentage of spermatozoa binding to the *zona pellucida* [Kordan *et al.* 1999].

Proteins belonging to the spermadhesin family have also been identified in the reproductive tracts of other animal species and humans. In cattle, investigations enabled the identification and characterization of three proteins belonging to the spermadhesin family, namely: BSP-A1/A2 (also referred to as PDC-109), BSP-A3 and BSP-30-kDa [Calvete *et al.* 1995b, Manjunath and Thérien 2002, de Cuneo *et al.* 2004]. In addition, BSP-like proteins have been identified in ram and buffalo [Manjunath and Thérien, 2002], as well as in stallion and human seminal plasma [Calvete *et al.* 1995bc].

As mentioned earlier, the mass spectrometry analysis of polypeptides 3 and 4 also demonstrated their affinity to a precursor of lysozyme C. The antibacterial properties of lysozyme, a cationic protein, are due to its ability to hydrolyze the glycosidic bond between N-acetylmuramic acid and N-acetylglucosamine in bacterial peptidoglycan. Moreover, lysozyme, occurring in almost all body fluids, binds to negatively-charged lipopolysaccharide through its polycationic domains, resulting in a suppression of its endotoxic activity [Ślęmp-Migiel and Wiczowski 2006]. Bielas [2002] demonstrated a positive interaction of lysozyme with the plasmalemma of boar spermatozoa, particularly at the mid-piece region. Further investigations by Bielas *et al.* [2003] demonstrated that the addition of 4 or 6 µg of lysozyme dimer to 1 ml fresh boar semen enhanced the motility of frozen-thawed spermatozoa, without having any marked effect on acrosome integrity. Moreover, fresh semen supplementation with lysozyme

had a beneficial effect on motility parameters and acrosome integrity of frozen-thawed stallion and dog spermatozoa [Dubiel *et al.* 2000, Niżański *et al.* 2000].

The results of this study indicate that the four conserved polypeptides of boar seminal plasma, analysed by mass spectrometry, showed similarity to spermadhesins and lysozyme C. This allows to anticipate that the polypeptides analysed might be implicated in the processes accompanying fertilization and in regulation of the male gamete activity. Finally, the findings of this study point to the significant role of secretions of the accessory sex glands and epididymis of boar in the regulation of these processes.

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Władysław Kordan, Agata Malinowska,
Marzena Mogielnicka, Marek Lecewicz

Zastosowanie spektrometrii mas (MS) do charakterystyki struktury wybranych polipeptydów plazmy nasienia knura uzyskanych po elektroforezie dwukierunkowej (2-D PAGE)

Streszczenie

Stosując metodę elektroforezy dwukierunkowej (2-D PAGE) zidentyfikowano w składzie map polipeptydowych plazmy nasienia knura cztery zakonserwowane polipeptydy o identycznej masie cząsteczkowej 24 kDa i następujących zakresach punktu izoelektrycznego: (1) 7,4-7,7; (2) 8,1-8,4; (3) 8,5-8,8 i (4) 9,2-9,4. W analizie ich struktury molekularnej zastosowano spektroskopię mas (LC – MS/MS). W prezentowanych badaniach, zastosowana po raz pierwszy komputerowa analiza widma masowego peptydów uzyskanych po trawieniu trypsyną wszystkich badanych polipeptydów, wykazała ich podobieństwo do rodziny spermadhezyn (*crystal structure of two members of the spermadhesin family*), zwłaszcza do spermadhezyny najądrzowej – AWN-1. Dodatkowo wykazano homologię polipeptydów 3 i 4 z prekursorem lizozymu C (*1,4-beta-N-acetylmuramidase C*). Prezentowane wyniki sugerować mogą udział analizowanych polipeptydów w procesach towarzyszących zapłodnieniu.