

# Immunodominant Semen Proteins II: Contribution of Seminal Proteins to Female Immune Infertility

(IgG antibodies / female immune infertility / prostate-specific antigen / prostatic acid phosphatase)

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**Abstract.** Seminal fluid is a protective medium for sperm, but it also represents potential immunogenic structures for the female immune system. Anti-seminal antibodies may threaten early fertilization. The aim of our work is to detect and identify seminal proteins that are related to female isoimmunization. In this report, we quantified serum anti-seminal IgG antibodies. Seminal proteins were analysed by two-dimensional gel electrophoresis followed by immunoblotting. To identify IgG-binding proteins of interest, a proteomic approach was selected. The dominant seminal antigens were detected within the relative molecular mass ranging from 25 to 85 kDa and the isoelectric point from 5 to 7. The detected proteins

were further identified as prostate-specific antigen, prostatic acid phosphatase, zinc- $\alpha$ -2-glycoprotein and zinc finger protein 778. Since these proteins were recognized by IgGs produced by infertile women and not by fertile women, we presume that major seminal antigens may play an important role in the pathogenesis of female immune infertility. Our study suggests the pattern of seminal proteins for further therapeutic attempts in the diagnosis of female immune infertility.

## Introduction

Seminal fluid (SF) represents a part of semen containing a range of organic/inorganic substances that are necessary for the physiological metabolism of sperm. It also acts as a transport, diluent and buffering medium (Kumar et al., 2009; Brázdová et al., 2012a). SF includes a repertoire of signalling molecules interacting with epithelium in the female reproductive tract. Local reactions may lead to inflammation (Robertson, 2005). SF has a built-in mechanism preventing immunological sensitization of the female against sperm as well as seminal structures. This protective system exists due to the presence of immune inhibitors originating in the male sex accessory glands (Prakash, 1981). SF has thus been suggested to be the modulator of sperm-induced inflammation (Troedsson et al., 2005), although in most cases SF prevents sensitization.

Some studies showed that the female fertility potential can be altered as a result of antibody formation against sperm antigens. The antibody fraction reacting with seminal antigens targets most of seminal proteins adsorbed on the sperm. SF induces the recruitment of macrophages and dendritic cells into cervical and endometrial tissues. Vaginal isoimmunization against any se-

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Abbreviations: 2D SDS-PAGE – two-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis, ACN – acetonitrile, AEC – amino-ethylcarbazole, AP – alkaline phosphatase, DHB – dihydroxybenzoic acid, ELISA – enzyme-linked immunosorbent assay, HRP – horseradish peroxidase, IEF – isoelectric focusing, IgE/G – immunoglobulin E/G, MALDI-TOF – matrix-assisted laser desorption/ionization-time of flight, Mr – relative molecular mass, NC – nitrocellulose membrane, PAP – prostatic acid phosphatase, pI – isoelectric point, PSA – prostate-specific antigen, SF – seminal fluid, TFA – trifluoroacetic acid, WHO – World Health Organization, ZAG – zinc- $\alpha$ -2-glycoprotein, ZNF778 – zinc finger protein 778.

men component can impair fertility. On the other hand, SF participates in the remodelling of cervical tissue to enable embryo pre-implantation. SF has also an important role in fertilization and embryonic development (Ulčová-Gallová, 2006; Robertson, 2007; Brázdová et al., 2012b). So far, only few seminal antigens linked to the female immune response have been characterized and determined. SF has rather been involved in the rare IgE-mediated reaction to semen (Weidinger et al., 2006). In this case of hypersensitivity, patients have difficulties to conceive, but infertility has not been demonstrated. SF is investigated with the purpose to evaluate pathological spermograms and to monitor the progression of prostate cancer by the level of prostate-specific antigen (PSA) and prostatic acid phosphatase (PAP) (Jones, 1991; Ostrowski and Kuciel, 1994).

The aim of our study was to describe seminal antigens and to identify various patterns interacting with serum IgG antibodies obtained from women diagnosed with fertility failure. We identified four antigens using mass spectrometry based on the proteomic approach. Our results brought evidence of SF sensitization and its connection with female isoimmunization and not only with semen hypersensitivity. We provided a significant basis for the profiling of infertile female patients.

## Material and Methods

### *Sample preparation*

Semen samples from eight normozoospermic (WHO, 2010) healthy donors (the average age of the voluntary donors was 27 years) were obtained by masturbation after 3–5 days of sexual abstinence. A mixture of protease inhibitors (Sigma Cocktail, Sigma-Aldrich, St. Louis, MO) was added to the ejaculates. SF was separated from sperm by centrifugation at  $1075 \times g$  for 15 min at 4 °C. The SF supernatants were pooled to increase the amount of potential proteins/antigens. Protein concentration was measured according to Smith et al. (1985). The samples were stored at -20 °C until assayed. All experiments were performed after obtaining informed written consent.

### *Patients*

Patient sera were obtained from 30 women with fertility failure (patients with repeated *in vitro* fertilization failure, the average age of the women was 35 years) and 10 from fertile women (control group, the average age of the women was 33 years) with proven fertility (one or two children). The equal volume of each serum was pooled to obtain sufficient volumes of patient and control serum. The aliquots of pools were frozen at -20 °C until assayed. This study was approved by the institutional ethical committees and informed written consent was obtained from the female infertile patients and female fertile controls.

### *Quantification of serum anti-seminal IgG antibodies*

Female serum IgG antibodies linked to SF proteins were quantified by ELISA method. In the first protocol, to evaluate and quantify total serum IgG, the microplates (MaxiSorp™, Nalge Nunc International, Roskilde, Denmark) were coated with anti-human IgG (whole molecule, Sigma Aldrich) in 50 mM carbonate-bicarbonate buffer (0.2 M Na<sub>2</sub>CO<sub>3</sub>, 0.2 M NaHCO<sub>3</sub>; pH 9.64) overnight at 4 °C. The plates were saturated with 0.5% gelatine (type B, Sigma-Aldrich) in PBS-Tw 0.1% (0.14 M NaCl, 8.1 mM Na<sub>2</sub>KPO<sub>4</sub>·12 H<sub>2</sub>O, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, 2.7 mM KCl, 0.1% v/v Tween 20; pH 7.4) for 2 h at room temperature. The coated and saturated wells were incubated with the individual patient or control serum or human IgG (Fc receptor specific, Sigma-Aldrich) of known concentrations in the serial dilutions for 2 h at 37 °C in PBS-Tw 0.1% and then with alkaline phosphatase (AP)-conjugated goat anti-human IgG (Fc receptor specific; Sigma-Aldrich) for 2 h at 37 °C. The AP activity was detected by p-nitrophenyl phosphate disodium kit (Sigma-Aldrich). Optical density was measured at 405 nm versus 630 nm. The wells were rinsed with 0.9% NaCl-Tw 0.1% after each incubation step.

In the second protocol, the SF reactivity of patient and control sera was tested by ELISA in an indirect non-competitive format. The micro-plates were coated with 1 µg/well of SF in a 50 mM carbonate-bicarbonate buffer overnight at 4 °C. The plates were saturated with 0.5% gelatine in PBS-Tw 0.1% for 2 h at room temperature. The coated and saturated wells were then incubated with the patient or control sera for 2 h at 37 °C in PBS-Tw 0.1% and then with AP-conjugated goat anti-human IgG for 2 h at 37 °C. The AP activity was detected by p-nitrophenyl phosphate disodium kit. Optical density was measured at 405 nm versus 630 nm. The wells were rinsed three times with 0.9% NaCl-Tw 0.1% after each incubation step. The anti-seminal IgG concentration was obtained by linear regression in comparison with the standard IgG calibration curve obtained in the first protocol.

### *Two-dimensional polyacrylamide gel electrophoresis (2D SDS-PAGE)*

Isoelectric focusing (IEF) was performed in the ReadyPrep IPG Strips pH 3–10 (Bio-Rad, Mississauga, ON). After IEF, the strips were equilibrated according to the manufacturer's instructions (ReadyPrep 2D Starter Kit, Bio-Rad), then put on 10% acrylamide SDS gel and overlaid with melted agarose (ReadyPrep 2D Starter Kit, Bio-Rad). The separated proteins were either transferred onto a nitrocellulose sheet (NC, 0.45 µm pore size, Serva, Heidelberg, Germany) or silver-stained (Silver™ Plus Stain Kit, Sigma-Aldrich) to visualize the proteins, or Coomassie-stained (Coomassie Brilliant Blue R-250, Sigma-Aldrich) to excise the spots of interest as it is a suitable indication for mass spectrometry.

## Immunoblotting analyses

Western blot analyses were performed as described (Towbin et al., 1979). The membrane was saturated with PBS-Tw 0.3% (PBS, 0.3% v/v Tween 20; pH 7.4). NC sheets were incubated with the serum pool overnight at 4 °C, then with horseradish peroxidase (HRP)-conjugated anti-human IgG (Fc specific, Sigma-Aldrich) for 2 h at 20 °C. The HRP activity was detected by 3-amino-9-ethylcarbazole substrate (AEC, Sigma-Aldrich).

## 2D image digitizing

2D images were digitized using the program ImageJ to evaluate the density/intensity of identified spots. The pictures were processed at 740×575 pixels, converted into 3D format and digitized at 660×600 pixels at RGB grey scale level depth. The intensity was calculated using the Micro Array Profile tool.

## Mass spectrometry determination

The protein spots of interest were analysed by the MALDI-TOF method. IgG-binding proteins revealed by immunoblotting were excised from Coomassie-stained gels. The spots were destained by 100 mM ammonium bicarbonate/acetonitrile ( $\text{NH}_4\text{HCO}_3/\text{ACN}$ , 1:1, v/v). The proteins were reduced (10 mM dithiothreitol, 45 min, 56 °C), alkylated (55 mM iodoacetamide, 30 min, 25 °C) and digested (trypsin [Promega, Madison WI], 3 h, 37 °C). The particular peptides were extracted from the gel by 35% ACN supplemented with 0.1% trifluoroacetic acid (TFA) and by 70% ACN with 0.1% TFA. The extracts were lyophilized, resolved in 0.1% TFA, desalted using ZipTip  $\text{C}_{18}$  pipette tips (Millipore, Bedford, MA), mixed with the matrix solution (1:1, v/v) and placed on the steel target plate. The matrix (2, 5-dihydroxybenzoic acid, DHB) was used at the concentration of 15 mg/ml in 33% ACN supplemented with 0.1% TFA. The samples were measured using the Biflex IV mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a UV nitrogen laser (337.1 nm, Laser Science, Franklin, MA). Spectra were measured in a reflection mode (mass interval 0.7–4 kDa). The Bruker peptide calibration standard II (Bruker Daltonics) was used for external (next-spot) calibration. Spectra were recalibrated using tryptic peaks as internal calibration. The raw data were processed by the mMass program (Strohalm et al., 2008). The proteins were identified using the Mascot search engine (Perkins et al., 1999) within the SwissProt database (Swiss-Prot Protein Knowledgebase online).

## Results

The individual SF samples from eight donors were pooled to cover the complex spectrum of seminal proteins/potential antigens. The protein concentration of the SF pool ranged from 20 to 25 mg/ml. Figure 1 shows the concentration of seminal-specific IgG antibodies in the individual sera of infertile and fertile women. IgG

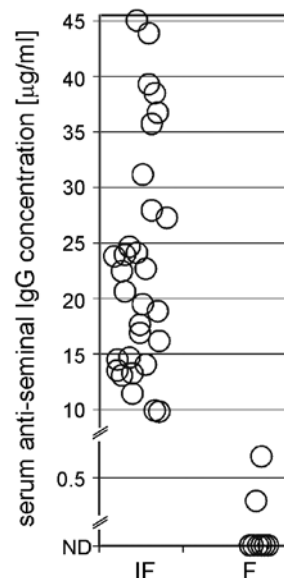


Fig. 1. ELISA quantification of female serum IgG antibodies linked to SF proteins. IF: infertile female patients, F: fertile female controls, ND: non-detectable level.

antibodies linked to seminal proteins were detected in all 30 sera of infertile females, ranging from 9.5 to 45 µg/ml. In comparison, the anti-seminal protein IgG level was detectable only in two sera of fertile controls out of 10, 0.32 and 0.66 µg/ml, respectively. Figure 2 illustrates 2D electrophoretogram (Fig. 2A) and IgG immunoblotting analyses (Fig. 2B, C). The SF profile (Fig. 2A) provided the spectrum of proteins ranging from 25 to 100 kDa and isoelectric point (pI) from 5 to 9. By comparison of 2D gels with their 2D blots, we found antigens recognized by the serum pool from female infertile patients (Fig. 2B). The most intensive spots were identified by MALDI-TOF as a zinc finger protein 778 (ZNF778, Fig. 2A, spot 1), prostatic acid phosphatase (PAP, Fig. 2B, spot 2) and prostate-specific antigen (PSA, Fig. 2A, spot 4). Another protein was weakly detected by the immunoblotting of SF and was further identified as zinc- $\alpha$ -2-glycoprotein (ZAG) (Fig. 2B, spot 3). Other patterns of seminal antigens showing Mr of 30–35 kDa, pI 5.6 and then Mr of 117–130, pI 5.7, were detected on blots but not identified with a sufficient mass spectrometry score. No seminal IgG-binding proteins were detected using the control serum pool (Fig. 2C). The digitized signal of seminal proteins/antigens is shown in Figure 3A and B, respectively. We conclude, based on 2D histograms, that the identified proteins are major antigens detected by the infertile patient serum pool. Interestingly, all isoforms of the immunodominant SF proteins were detected as the antigenic patterns. The biochemical attributes and parameters of the above-mentioned proteins are summarized in Table 1.

## Discussion

Failure of natural tolerance may over-stimulate the immune system towards SF or sperm. Female IgG anti-

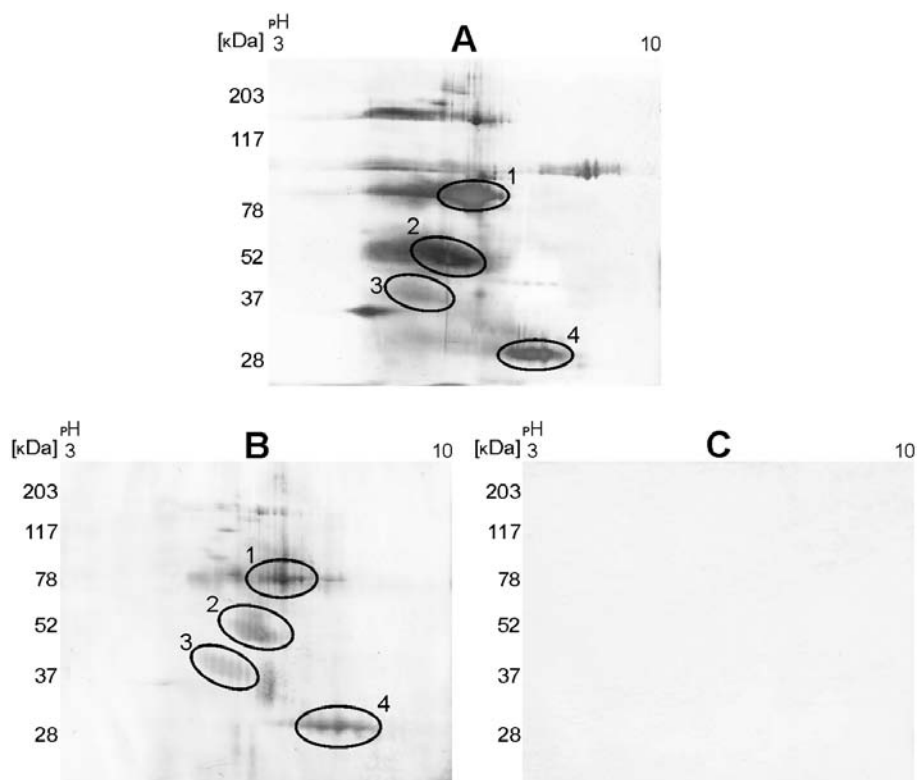


Fig. 2. Seminal proteins separated by 2D SDS-PAGE either silver-stained (A) or immunoblotted (B, C). B: immunodetection using the serum pool of infertile women, C: immunodetection using the serum pool of fertile women, 1–4: antigens identified by mass spectrometry.

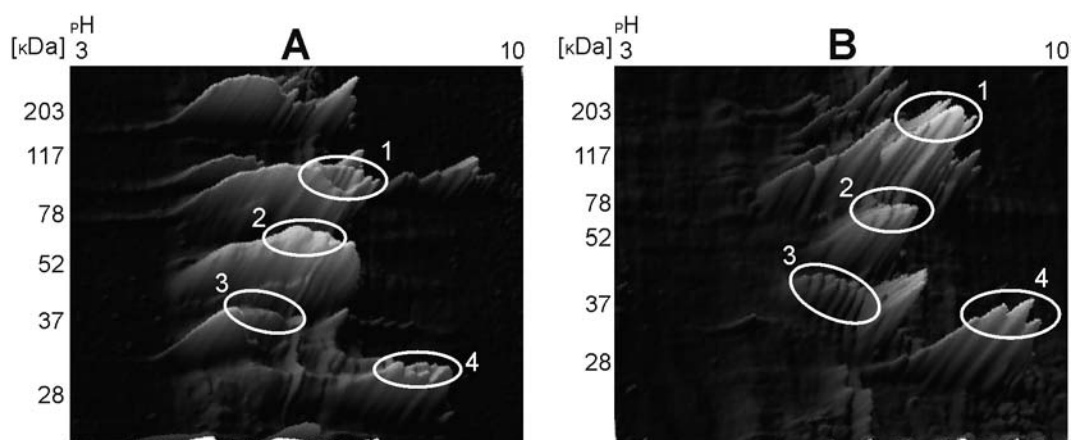


Fig. 3. Quantification of signal revealed in 2D images. A: quantified spots on silver-stained 2D gel, B: quantified spots on 2D blot, 1–4: antigens identified by mass spectrometry.

Table 1. Biochemical attributes and parameters of identified proteins

Protein	Spot	Mr [kDa]	pI	Score <sub>a</sub>	Protein Coverage <sub>b</sub> [%]	Integrated density on 2D gel [%]	Integrated density on 2D blot [%]
Zinc finger protein 778 (ZNF778)	1	85	5.8	84	17	35.7	38.1
Prostatic acid phosphatase (PAP)	2	45	5.6	92	35	29.6	33.2
Zinc- $\alpha$ -2-glycoprotein (ZAG)	3	35	5.6	189	54	21.5	29.4
Prostate-specific antigen (PSA)	4	29	6.7	156	56	28.3	30.8

<sup>a</sup> Protein score was evaluated by the Matrix search engine as  $-10 \cdot \log(P)$ , where the value P was the probability (Perkins et al., 1999). Protein score greater than 56 was considered as significant ( $P < 0.05$ ).

<sup>b</sup> Protein coverage was calculated as the ratio of amino acid number among the detected peptides to the amino acid number of each protein.



bodies act on the SF when the ejaculate is deposited into the vagina. IgG antibodies against SF proteins have been described and were suggested to be involved in female immune infertility (Brázdová et al., 2012a). The repertoire of reactions leading to the activation of inflammatory cytokines and leukocyte infiltration is wide. Which seminal protein activates these reactions has not been pointed out yet (Starita-Geribaldi et al., 2001; Sharkey et al., 2007; Plessis et al., 2011). Our results represent ongoing study of seminal proteins (Brázdová et al., 2012a) relevant to antibody formation in infertile women. In this report, we quantified the concentration of anti-seminal IgGs in fertile and infertile women. Then, we identified the isoantigens recognized by the serum pool of infertile women.

On the basis of previous studies (Rodriguez-Martinez et al., 2011; Brázdová et al., 2012a) and the fact that men might differ in their SF content, we decided to prepare a pool of seminal fluids from eight healthy normozoospermic (WHO, 2010) donors to extend the potential repertoire of IgG-binding proteins by increasing the heterogeneity of the sample. In order to characterize individual SF proteins and to identify the located seminal antigens from a complex SF mixture, we used a proteomic approach based on 2D immunoblotting and mass spectrometry. Our experiments using two different methods – quantitative (ELISA) and semi-quantitative (immunoblotting) – show that male seminal proteins are able to trigger antibody production in the female patients diagnosed with immune infertility. Using the control serum pool from fertile women, no IgG binding with seminal antigens was detected on the immunoblot. This is in agreement with ELISA results showing that only two fertile women out of 10 show a very low level of anti-SF-reactivity. Such differences in IgG concentration and specificities between fertile and infertile women support the hypothesis of the anti-seminal IgG antibodies involved in the pro-inflammation process preventing successful fertilization.

Seminal proteins contribute to male fertility and are essential in several steps of fertilization (Qu et al., 2007). In order to characterize the proteins that could be the targets of antibodies in infertile women, we kept the seminal samples untreated to maintain, as much as possible, their native form. This fact could explain the smears on 2D images (Fig. 2A and B). The optimal solubility of the SF extract would be achieved using a mixture containing urea, thiourea and CHAPS (Starita-Geribaldi et al., 2001). This mixture is considered to keep the entire spectrum of epitopes that might be involved in the elicitation of pro-inflammatory circuits in the female reproductive tract.

SF is mostly associated with semen hypersensitivity. Particularly, PSA has been suggested to be linked to allergic reaction or anaphylaxis involving IgE antibodies. PSA and one isoenzyme of acid phosphatase, PAP, are used to monitor and assess the progression of prostate cancer and are usually also related to a pathological spermogram (Jones, 1991; Ostrowski and Kuciel, 1994;

Weidinger et al., 2006; Hassan et al., 2008a). In the current study, both markers may have indicated the activated humoral immunity of infertile females since they were identified as the immunodominant antigens. As it was previously mentioned, PSA is thought to be associated with the rare phenomenon of semen hypersensitivity inducing specific IgE antibodies; however, we have shown that PSA is also able to induce IgG antibodies in infertile women. ZNF778 is a nuclear protein that has been described at the nucleic acid level in various tissues including testis (Ota and Suzuki, 2004). ZNF778 is considered, for the first time to the best of our knowledge, as an IgG-binding protein that may be involved in antibody formation during the pathological isoimmunization. ZAG has been mentioned as a sperm antigen and suggested to have a role in the expression of the immune response (Lilja et al., 1987; Cross, 1996). Our results are in agreement with this observation and we confirmed that ZAG is an IgG-binding protein even though we found it in the SF. This localization could be explained by the fact that ZAG possibly complexes with other seminal structures (Hassan et al., 2008b).

We conclude that (1) 100 % of women, selected upon infertility criteria, express IgG antibodies against SF proteins and (2) at least three SF proteins, namely PSA, PAP and ZNF778, are related to the pathologic immune response associated with female sensitization. SF proteins are thus involved not only in the IgE-mediated semen hypersensitivity. The herein described proteins might be considered as biomarkers of such pathology.

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