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Molecular Microheterogeneity of Prostate Specific Antigen in Seminal Fluid by Mass Spectrometry

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Abstract

Objectives—Prostate specific antigen (PSA) is a widely used and clinically valuable marker for prostate disease. In order to enable the development of new PSA assays and progress the understanding of the biology of PSA we have analyzed PSA in seminal plasma.

Design and Methods—PSA in seminal plasma from men attending a fertility clinic and healthy controls was analyzed using SDS-PAGE, Western blotting and mass spectrometry.

Results—Using mass spectrometry, different forms of PSA could be identified in 1–9 bands seen on SDS-PAGE analysis of the respective sample. However, a majority of these molecular forms of PSA were not observed on Western blots. Enzymatic activity of PSA isoforms was demonstrated by sequencing data in zymogram gels. Multivariate analysis of clinical data revealed well-separated patient groups.

Conclusions—We demonstrated that PSA in seminal plasma occurs in several isoforms, yet not all were detectable using an antibody based clinical routine method. The heterogeneity of PSA expression might be of clinical significance, by an improved patient phenotyping.

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Conflict of interest

Dr. Hans Lilja holds patents for free PSA and kallikrein-2 (hK2) assays. All other authors declare no conflict of interest.

Keywords

prostate specific antigen; isoform; seminal plasma; infertility; MALDI LTQ Orbitrap XL; ESI-LTQ FT-ICR

1. Introduction

Prostate cancer is the most common cancer in Sweden (8,870 new cases in 2007) accounting for approximately one third of all cancer diagnoses among men (Socialstyrelsens Cancerregistret 2009, <http://www.roc.se/cancerreg.asp>). The worldwide prevalence is increasing and approximately one out of six men will be diagnosed with prostate cancer. The clinical course of the disease varies and the majority of the patients will eventually die of other causes than prostate cancer. Quantitative analysis of the prostate specific antigen (PSA) in plasma is a corner stone both for diagnosing and monitoring the disease [1; 2]. Approximately, 25 million PSA tests were performed worldwide in 2005 (Clinical Data, Inc., Newton, MA, <http://www.clda.com>).

Although, PSA is the clinically most valuable tumor marker used, it is associated with considerable specificity and sensitivity problems. Increased PSA values can result from malignant as well as benign prostate disease, *e.g.*, hyperplasia or prostatitis. Consequently, 65–75% of men who undergo a prostate biopsy due to a moderate PSA elevation (≈ 3 –10 ng/mL, ref. value < 3 ng/mL) do not have evidence of cancer [3; 4] and 25% of men with PCa have normal PSA levels [5]. To improve the specificity, two molecular forms of PSA are routinely analyzed: free PSA (fPSA) and total PSA (tPSA = the sum of fPSA and PSA in complex with α_1 -antichymotrypsin, SERPINA3). The level of fPSA is lower in men with malignant disease than in men with benign hyperplasia. Yet, there is no clear-cut method to distinguish the various forms of prostate disease.

In order to improve its clinical value, new immunoassays for different molecular forms of PSA, *e.g.*, pro-PSA, intact PSA and BPSA (a PSA variant internally cleaved at Lys182/Ser183 [6; 7]) have been developed but so far no substantial increment in diagnostic accuracy compared to analysis of tPSA and fPSA [8] has been demonstrated.

PSA is a serine protease produced by the epithelial cells of the prostate and secreted as an inactive proenzyme (proPSA) into seminal fluid [4; 9], where it can be activated by the kallikrein-related peptidase 2 (hK2) and other endopeptidases of the prostate [4; 10–12]. PSA has restricted chymotrypsin-like endoproteolytic activity, cleaving its biological substrates, semenogelin 1 and 2 (SEMG1; SEMG2) [13; 14], and fibronectin [13], as well as laminin and gelatin [15]. PSA in seminal fluid occurs predominantly in an active single-chain form. A minor fraction is inactivated due to internal cleavages [9; 12] or complex formation with the protein C inhibitor (SERPINA5), released from the seminal vesicles [16–18]. Furthermore, it has been demonstrated that fPSA displays a considerable structural heterogeneity in serum, seminal plasma, and hyperplastic or cancerous tissue [19]. It has been also recently reported that men with male factor infertility have an increased risk of subsequently developing aggressive high-grade prostate cancer [20]. For this reason, it has been suggested that male infertility may be an early and identifiable risk factor for the development of clinically significant prostate cancer.

Posttranslational modifications of the PSA molecule also contribute to the structural heterogeneity. PSA is a glycoprotein composed of approx. 8% *N*-linked carbohydrate [21], a biantennary *N*-linked oligosaccharide of the *N*-acetylglucosamine type with terminal sialic acid groups [22]. PSA in seminal fluid displays large heterogeneity mainly because of a

variable degree of sialylation [23]. It has been shown that the carbohydrate side chains differ greatly in plasma and seminal fluid in PCa patients [24]. Contributing to the heterogeneity of the PSA molecule there are also differences in primary structure, carbohydrate composition and enzymatic activity [7; 23; 25–30].

Exactly which molecular form(s) of PSA the different commercially available clinical routine assays measure has not been fully elucidated. Differences in the composition of the PSA molecular forms could reflect diversity in the biology of prostate disease, which might be of diagnostic value.

We have previously presented a strategy, that the combination of analytical principles can improve the resolving power of PSA identification, complementarily utilizing 1-D gel electrophoresis and high resolution MALDIMS, which could confirm complex patterns of PSA forms in seminal plasma [31]. In the present study we have applied these technology platforms, in addition with clinical data, to characterize the expressed molecular forms of PSA in seminal plasma. Thirty-four participants were enrolled in this study with both healthy controls and patients, *i.e.*, men being diagnosed for infertility. Our study objective was to employ modern proteomic tools to characterize different expression of PSA variants in seminal fluid. Taking into consideration the recent reports on the disease link between male infertility and prostate cancer [20], the study outcome opens up for functional interpretations related to PSA isoform expression patterns and clinical demography.

2. Material and Methods

2.1. Seminal plasma

Semen samples were obtained from young men undergoing investigation for infertility prior to final diagnosis of disorders and healthy volunteers. Seminal plasma was provided by the Center for Reproductive Medicine at Malmö University Hospital, following the guidelines of the Helsinki Declaration. The collection of seminal plasma was approved by the ethical board at Lund University (approval number: LU 532-03) and the samples were processed according to the WHO guidelines (WHO, 1999). Seminal plasma was prepared by centrifugation at 10,000 *g* for 10 min and stored at -20°C until use. Table 1 gives details on the clinical data from the participating subjects in the study. Free PSA (fPSA) ranged between 0.233 and 1.915 mg/mL (see Table 1) as determined by the DELFIA assay (Perkin Elmer, Turku, Finland) [32]. The total protein content of seminal plasma samples was determined using Bradford reagent (Sigma, Steinheim, Germany) and equal amounts of protein (88 μg) were applied on gels. The semen volumes are aligned with age matching and the PSA levels measured by ELISA (DELFI). In addition, we analyzed the fructose levels, because patients with obstruction or aplasia of vas deferens have typically low fructose concentrations. Clinical implication of impaired prostate function is often associated with low zinc levels, whereas the inflammatory status represented by various inflammatory processes (*e.g.*, prostatitis) results in an increased albumin level. Correspondingly, PSA, fructose, zinc and albumin assays were completed and these data are presented in Table 1.

2.2. Gel electrophoresis techniques

PSA expression was detected by SDS-PAGE gel electrophoresis in 0.75 mm thick, 12 %T, 2.67 %C polyacrylamide gels under reducing conditions. The samples were reduced with 4 mM DL-dithiothreitol while boiled for 5 minutes and alkylated by addition of 2 μL of 0.5 M iodoacetamide at room temperature for 15 min. Following preparation 10 μL of samples and 3 μL of protein molecular weight standards (PageRuler™ Prestained Protein Ladder Plus from Fermentas, St. Leon-Rot, Germany) were loaded. The gels were run at a constant voltage of 125 V for 1 h 10 min in a MiniProtean III electrophoresis unit (Bio-Rad,

Hercules, CA). Staining was performed with Coomassie blue R350 (GE Healthcare, Uppsala, Sweden) for 1 h according to the manufacturer's recommendations.

The Western blot analysis was performed using 0.75 mm thick, 12 %T, 2.67 %C polyacrylamide gels run at non-reducing conditions. The separated protein bands were transferred onto PVDF membranes (0.45 pore size Invitrolon™ PVDF, Invitrogen, Carlsbad, CA) using a tank blotting (Bio-Rad, Hercules, CA) at constant current (350 mA) for 1.5 h. The membranes were blocked with 1% of BSA in 50 mM Tris-HCl/150 mM NaCl/0.1% Tween-20, pH 7.4. The primary antibodies (2E9) [33] were used at 1:5000 dilution. The secondary antibody of ECL anti-mouse IgG (used at 1:10000 dilution) and the horseradish peroxidase reagent system (ECL) were purchased from GE Healthcare (Uppsala, Sweden). Enzymatic activity of PSA forms was monitored by zymogram gel electrophoresis using non-boiled sample aliquots in 0.75 mm thick, 12 %T, 2.67 %C polyacrylamide gels containing 0.1% gelatin from porcine skin (Sigma, Steinheim, Germany) under non-reducing conditions in the presence of SDS. In order to refold the proteins allowing re-gain their enzymatic activity, the detergent was removed by washing with Triton X-100 and the gels were incubated overnight at 37°C in Zymogram Development Buffer (Invitrogen, Carlsbad, CA). Staining was performed with Coomassie blue R350 for 1 h according to the manufacturer's recommendations.

The molecular weights of proteins were calculated by comparing their migration distances to the positions of molecular weight standards, using Quantity One software (Bio-Rad, Hercules, CA). Further on, the major form of PSA (ca. 32 kDa), identified in all samples, was used for normalization of molecular weights of proteins in the gels.

2.3. Mass spectrometry

The major protein bands observed within the size region between 20–40 kDa in SDS-PAGE gels were cut and analyzed by MALDI mass spectrometry following in-gel tryptic digestion. All analyses were performed in triplicates. Full mass scans and MS/MS spectra were generated on a MALDI LTQ Orbitrap XL instrument (Thermo Scientific, Bremen, Germany) using two acquisition methods consecutively on each spot. First, the FT mass analyzer (Orbitrap) of the mass spectrometer was utilized for full MS data collection in positive mode within a mass range of 600–4000 Da at 60,000 resolution. Laser energy was set to 10 µJ. Each dry droplet spot was sampled by acquiring 20 FT mass scans in 2 microscan/scan steps, allowing the software to select positions automatically based on the matrix crystals (Crystal Positioning System). The monoisotopic masses were extracted from the averaged spectra by using the built-in Extract script of the Xcalibur software v2.0.7.

The second method for peptide sequence data collection utilized the linear ion trap mass analyzer of the hybrid instrument. The data acquisition was performed on the 25 most intense signals, which exceeded the minimal intensity of 500 counts and matched with the inclusion mass list theoretically created by *in silico* digestion of 35 PSA isoforms using the PeptideMass tool (<http://expasy.org/tools/peptide-mass.html>). For fragmentation the normalized collision energy was set to 50% and the activation *Q* to 0.250 for an activation time of 30 ms.

In-gel digests of enzymatically active proteins within the size region of 20–40 kDa in zymogram gels were analyzed by nanoLC-MS/MS. An HTC-PAL autosampler (CTC Analytics AG) was employed for 2 µL injection of samples, which were first trapped on a 4.5 cm long C₁₈-precolumn (100 µm i.d.). Then reversed-phase separation of peptides was performed on a 20 cm long fused silica column (50 µm i.d.) packed with ReproSil-Pur C₁₈-AQ 3 µm porous particles (Dr. Maisch GmbH, Germany). The linear gradient started after 6 min of isocratic run at 5% ACN/0.2% formic acid and reached 50% ACN in 34 min at 200

nL/min flow rate using a binary pump (Agilent 1100) with splitter. The LTQ FT-ICR (Thermo Electron), a hybrid mass spectrometer equipped with linear ion trap, Fourier transform ion cyclotron (7 T magnet) was operated in data dependent mode switching between MS and MS/MS acquisitions automatically. While the FT-ICR mass analyzer acquired survey MS spectra in the range of 400–2000 Da, the linear ion trap was used for fragmentation and detection of the five most abundant ions selected from each FT-MS scan. The inclusion mass list of PSA peptides was used for parent ion selection but other precursors were also fragmented when no masses from the inclusion list were present. Additionally, an exclusion list of masses originating from the background proteins (mostly gelatin) in zymogram gels was applied. The exclusion time for isobaric precursor ions was 20 s and at least 1000 counts were required for fragmentation. The normalized collision energy was set to 50% and the activation Q to 0.250 for an activation time of 30 ms.

2.4. Data analysis

Protein identification based on high resolution MALDI-MS data was performed by peptide mass fingerprinting (PMF) using extracted mass lists on the Mascot search engine (<http://www.matrixscience.com>) with the NCBI database (release 20091024, 224,815 out of 9,937,670 sequences), which contained the highest number of PSA isoforms. The parameters of fixed carbamidomethylation and variable oxidation modifications at cysteine and methionine residues were used respectively. The peptide tolerance of 10 ppm was used throughout database search. Positive PSA annotations by PMF were considered only when at least two specific peptides were identified in at least two of the technical triplicates.

The Proteios Software Environment (ProSE) [34] was employed for combining multiple search results using MALDI-MS/MS sequencing data, which is provided by the built-in interfaces of ProSE to protein identification engines [35]. We have simultaneously used both Mascot and X!Tandem with native k-score scoring, searching the IPI Human database [36] version 3.71 (containing 86,745 protein sequences). The precursor mass tolerance was set to 10 ppm, whereas the fragment tolerance was 0.5 Da. The cysteine and methionine residues were modified with fixed carbamidomethylation and variable oxidation, respectively. The filtering criterion of protein identification was defined as at least two PSA peptides required with an FDR less than 0.01 [35].

The enzymatically active PSA forms were identified by the tandem mass spectra generated on FT-ICR, using Proteome Discoverer version 1.1 (Thermo Fisher Scientific) applying Mascot search engine on the SwissProt database (March 2010). The database interrogation parameters were set to include the same mass tolerance windows and amino acid modifications as above but two missed cleavages were allowed.

3. Results and Discussion

We have analyzed seminal plasma samples from young men seeking medical aid due to infertility ($n=29$) and compared those to healthy controls ($n=5$). Seminal plasma was chosen in this study because of the fact that the major portion of PSA molecules is in free forms, which enhances the identification of possible PSA isoforms in this proximal fluid. Electrophoretic decomplexing and mass spectrometry based protein identification was employed according to our method reported previously [31]. As Figure 1 illustrates, the PSA expression profiles of clinical samples were determined by MALDI-MS generating accurate masses and peptide sequences, as well as the monitoring by Western blot analysis. Additionally, in this study we have extended the use of high resolving MS (FT-ICR), resulting in unambiguous protein annotations in zymogram gel bands, which has enabled us to identify enzymatically active PSA isoforms even in the presence of a high chemical background.

Both the free PSA (fPSA) and total protein concentrations of seminal samples were determined by commercial DELFIA and Bradford methods, respectively. The fPSA values were in the range of 0.233–1.915 mg/mL, whereas the total protein concentration varied between 7.715 and 22.097 mg/mL (see Table 1). The 34 individual samples revealed weak correlation between the level of total protein and PSA expression (correlation coefficient=0.62). With the study n-number, we were not able to show statistically significant differences in-between total protein- and fPSA-expressions within the two study groups, as shown in Figure 2A. The mean fPSA expression (relative to the total protein levels) was found to be 5% (Figure 2B), whereas the median value was 4.4% (minimum=2.0% and maximum=10.4%). Interestingly, four samples clearly deviated from average in the region of high fPSA expression (above 8%), including one healthy control sample. The other four control samples scattered around the median value.

3.1. Evaluation of PSA forms in clinical samples

Following calculation of the molecular weights (Mw) of protein bands relative to the molecular standards, these values were corrected within each gel on a band of unknown protein at about 44 kDa present in all samples. Further corrections were made in between gels, modifying the calculated Mw values by the difference of the reference band of the unknown protein band relative to 44.07 kDa (variation was less than 2.52 kDa). Since a major form of PSA (31.6 kDa) was clearly identified in all samples, this band was used for final normalization of all PSA bands. Protein bands in the higher size range (30 kDa and above) agreed excellently in all gels, but those bands at lower positions (below 25 kDa) scattered more in their calculated Mw values due to the more pronounced diffusion. Therefore, their optical detection was more difficult, which contributed to a less reliable determination of their molecular weights.

3.2. PSA expression in seminal fluid

In gel bands under reducing conditions, we could unambiguously identify single or double PSA forms at very close positions in 53% of the samples, combining accurate mass (FT full MS) and sequence (MS/MS) based database search, marked by asterisks in Figure 3 (samples SP5, SP6, SP8–16, SP20, SP23, SP24, SP26–28, SPC3 and SPC5). In these cases, a comparison with a single PSA form, detected by monoclonal antibody 2E9 in Western analysis, agreed well with PSA form at 31.6 kDa. A detailed list of PSA identifications by both peptide mass fingerprint and sequences is provided in the Supplementary Table.

In the remaining of the samples (47%), more than 2 PSA forms could be identified by MS, where 3–9 molecular variants were observed (Figure 3). Although, in 2 of these cases Western analysis also indicated multiple PSA forms, the mAb 2E9 missed the clear-cut detection of a significant portion of PSA in the remaining 12 seminal plasma samples.

Since the sample volume applied on gels was normalized to the total protein amount (88 µg), the amount of fPSA varied in the range of 1.78–9.13 µg. We have observed that the higher the fPSA amount in gel the more molecular forms could be identified by MS. In 16 samples more than 2 forms were detected and the relative fPSA expression was above the median value (4.37%) in 12 out of 16, corresponding to 75% of these samples. In those samples, where more than 3 molecular forms of PSA were identified by MS (10 samples in total), 7 samples were within the highest 10 relative fPSA expression levels. On the other hand, in 12 out of 18 cases, where single or double forms of PSA were detected, the relative fPSA expression was below the median value. Interestingly, single PSA forms were detected in two samples (SP13 and SP15) with high relative PSA expression levels. Consequentially, as the strong correlation between the number of observed molecular forms and the amount

of PSA applied in gels indicated, more variants could be present in all samples, but the concentration being below the limit of detection.

3.3. Enzymatically active PSA forms

The zymogram separation pattern generated 3–6 enzymatically active protein bands that migrated to the same positions within a 20–40 kDa, range in each gel. An apparent similarity of the respective PSA band distribution was evident, although the calculated Mw-values were not identical with those determined in SDS-PAGE gels due to the different gel compositions. By comparing protein expression patterns, the corresponding protein band distribution (separated under SDS-PAGE and zymogram gel conditions), with major PSA forms at 31.6 and 35.0 kDa, were found to be identical. We also identified active double bands, in our zymogram images, at low Mw-positions (see bands at 23.9 and 25.4 kDa in Figure 4) that we identified as being highly similar to the 23–27 kDa region (SDS-PAGE), where only a single band appeared with a broad and diffused elution profile.

In an attempt to associate the observed enzymatic activity in the zymogram gels with PSA specificity, the unstained bands were subjected to PSA sequencing using the FT-ICR high accuracy mass spectrometer. The FT-ICR platform allowed us to identify PSA forms in the presence of high chemical background. As exemplified with two of the patient samples; SP21 and SP22, we were able to observe 6 enzymatically active bands. Out of these forms, the highest Mw position was detected as 35 kDa, and positively identified as the major form of PSA by FT-ICR with patient SP22 only. The weak activity band in patients SP21 did not allow a positive sequence identity of PSA. Interestingly, patient SP21 showed positive identity by both sequencing, and activity for additionally four PSA forms (29.5, 28.1, 25.4 and 23.9 kDa), whereas the 28.1 kDa PSA form was found to be expressed by patient SP22 as well. The strategy outlined above, allowed us to predict the combined effects of enzyme activity in patient samples with a target identity of PSA.

3.4. Correlation analysis

Due to the high resolving power of modern proteomic platforms, we were able to detect the highest number of PSA forms and sequence information that has been identified, as of today, describing a multitude of molecular forms of PSA in seminal fluid. We captured the clinical data of the participants in the study, where age, semen volume, and zinc, fructose and albumin levels were determined. These study data formed the basis for the statistical analysis. The study members were shown to group well in the isomap plot, which lays out the samples in space, revealing the network clustering (see Figure 5A). Figure 5B shows these separated sample groups as a heat map, where samples with high albumin levels (calculated to be above the third quartile of the data set) gathered in the Red group and to a less extent in the Green group. In addition, the majority of the samples with low fructose and zinc concentrations (which came clear below the first quartile of the data set) were found in the Orange group and to a less extent in the Red group (see Figure 5B). Interestingly, we found that the samples with low semen volumes in the Red group also have (found below the first quartile of the data set), yet only one patient in this group is identified above the third quartile of age. All of these hierarchical clustering analyses were performed using the Qlucore Omics Explorer v2.1. software.

In the second step of the statistical evaluation, we investigated the Red and Orange groups that proved to have the highest risk for male factor infertility and followed their positioning when the proteomic data were combined with clinical information. Accordingly, besides the clinical data, the levels of fPSA and total protein concentrations, the number of peptides, and the sequences by both MS and Western analysis were included as variables for a multi group comparison. The following variables remained in this hierarchical clustering analysis after

filtering by variance ($\sigma/\sigma_{\max}=0.27$): total protein and fructose concentrations, semen volume, age and three identifications of PSA. The resulting isomap and heat map representations revealed a formation of four groups that are displayed in Figure 5C and D. Remarkably, the patients with high albumin levels (Red group in Figure 5A and B) grouped well together in the Yellow group (see Figure 5C and D). These findings are in good correlation with the first analysis round, where the separation of these patients was found to sustain with a strong correlation to an ongoing inflammation. The group of patients with low fructose and zinc levels (Orange group in Figure 5A and B) was also clearly separated in the Green group (Figure 5C and D).

Interestingly, the samples in the Blue group typically had low semen volumes but without any correlations to additional risk factors. The control samples were grouped in the Red and Blue groups, with a clear separation from the patients with male factor infertility (in the Green and Yellow groups within Figure 5C). The peptide sequences as variables from both PMF and MS/MS identification with the major expressed PSA form (at 31.6 kDa) indicated that the number of identified tryptic peptides was one of the most important separating factors in this analysis. Further, detailed investigation of the identified PSA peptide sequences in these separated groups (Yellow and Green in Figure 5C and D) revealed the highest number of peptide annotations.

4. Conclusions

In our opinion it becomes increasingly evident that PSA as a single marker needs additional and higher resolution data for improved disease speciation and predictive outcome. Despite notable advances in our understanding of the molecular and functional details of PSA, there is still lacking consensus as to which may be the best strategy to obtain optimal clinical value from PSA-testing [19]. Consequently, our observation of various isoforms of PSA in patients reported in this communication can contribute to important insight in identification of disease-relevant heterogeneity of PSA, including transcriptional and post-translational modifications present due to various stages and causes of prostate disease. In order to get an in-depth understanding of the metabolism of PSA and its variety of modified forms in blood and semen we need to extend the investigation on clinically relevant material to gather sufficient statistical data and elucidate the clinical potential of profiling PSA isoforms.

Highlights

- > We employed multiple proteomic platforms to characterize molecular forms of PSA in seminal plasma.
- > Several PSA isoforms were identified by sequencing data, yet the monoclonal antibody, recognizing total PSA, could not detect all these forms.
- > We demonstrated that high resolution FT-ICR MS could be useful to identify enzymatically active forms of PSA.
- > Multivariate analysis of clinical data revealed well separating patient groups.
- > Patient samples with risk of male factor infertility were annotated with high peptide numbers of the major PSA form, in contra

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Non-standard Abbreviations

PSA	prostate specific antigen
PCa	prostate cancer
BHP	benign prostatic hyperplasia
tPSA	total PSA
fPSA	free PSA

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Figure 1. Illustration of the mass spectrometry-based proteomics strategy developed for identification of PSA forms in seminal fluid samples.

Figure 2.

(A) Distribution plot of seminal fluid for total protein vs. free PSA concentrations. Based on these quantitative values the patient and control groups cannot be separated. (B) The distribution of relative PSA expressions (to the total protein amount) depicted as box plot diagram. The average value was 4.9%, whereas the RSD was 41%.

Figure 3. Summarized data of molecular forms of PSA identified by both peptide fingerprint and sequences. The dark boxes indicate molecular forms of PSA as identified by both peptide mass fingerprinting and sequences from high resolution MALDI Orbitrap data.

Figure 4. Illustration of enzymatically active PSA forms in zymograms demonstrated with two examples as identified by high resolution FT-ICR mass spectrometry.

Figure 5.

Isomap and heat map representation of the classical hierarchical clustering applied on the data set generated with (A and B) 5 clinically relevant parameters separately and (C and D) in combination with proteomic data of 34 individual seminal fluid samples. Samples associated with risk factors of male infertility were identified in the Red and Orange groups (A and B), which were re-grouped in Green and Yellow upon combination with proteomic data (C and D).

Summarized data of seminal plasma samples used in this study. Patient samples are numbered 1–29, whereas healthy control samples are denoted as C1–C5.

Table 1

Sample ID	Age (year)	Semen volume (mL)	Protein (mg/mL)	Albumin (mg/mL)	Fructos (mM)	Zinc (mM)	fPSA (mg/mL)	Nr of PSA form in SDS-PAGE (PMF/Sequence)	Nr of PSA form in Zymogram	Nr. of PSA form in Western
1	28	4.1	10.879	1	12.1	1.6	0.580	4/6	2	1
2	39	2.8	12.142	0.6	10.2	2.2	0.725	4/3	n.a.	1
3	46	4.6	16.053	0.5	13.8	2.8	0.655	2/3	n.a.	1
4	34	2.5	14.311	0.9	9	1.4	0.590	3/2	1	1
5	35	6.5	19.697	1	27	2.9	0.425	1/1	n.a.	1
6	36	5	11.395	0.5	18.7	1.3	0.459	1/1	n.a.	1
7	36	5	13.013	0.5	20.1	1.6	0.505	3/2	4	1
8	42	5.2	11.626	0.8	6.6	0.9	0.462	1/1	2	1
9	38	5.1	10.755	0.5	14.4	0.8	0.324	2/2	n.a.	1
10	40	3.8	16.853	0.8	14.9	1.8	0.493	2/1	1	1
11	41	6	7.893	0.4	13	0.7	0.344	2/1	n.a.	1
12	38	6.3	12.639	0.4	9.5	1.1	0.414	1/1	n.a.	1
13	35	5.9	10.648	0.4	8.4	1.2	0.695	1/1	n.a.	1
14	39	2.2	21.511	1.5	7.4	3	1.035	2/2	n.a.	1
15	42	3.1	13.493	0.7	16	2.8	0.930	2/1	3	1
16	36	3.1	15.146	1.3	11	2.4	0.610	2/1	n.a.	1
17	30	2.2	13.688	0.7	6.3	1.8	0.815	3/1	n.a.	2
18	32	5.7	12.444	0.5	14.5	0.8	0.368	2/2	n.a.	1
19	34	2.5	21.511	1.1	2	4.2	1.915	4/6	n.a.	1
20	37	7.8	12.195	0.7	23.3	1.8	0.590	2/2	n.a.	1
21	33	7.1	7.715	0.2	6.5	1.6	0.800	7/8	4	1
22	37	4	19.537	0.8	14.3	3.6	1.265	3/5	2	1
23	36	7.8	11.519	0.5	11.8	0.9	0.233	2/2	n.a.	1
24	31	4	9.315	0.3	18	0.9	0.409	1/1	n.a.	1
25	37	4.2	16.568	0.5	11.9	3.4	1.140	4/1	n.a.	1

Sample ID	Age (year)	Semen volume (mL)	Protein (mg/mL)	Albumin (mg/mL)	Fructos (mM)	Zinc (mM)	fPSA (mg/mL)	Nr of PSA form in SDS-PAGE (PMF/Sequence)	Nr of PSA form in Zymogram	Nr. of PSA form in Western
26	41	4.3	18.364	0.6	9.5	2.9	0.755	1 / 1	1	1
27	37	3.2	8.568	0.6	14.1	1.9	0.310	2 / 1	2	3
28	42	3	20.142	0.4	15.6	1.9	0.499	1 / 1	n.a.	1
29	36	4.2	13.831	0.8	8.1	4	1.320	3 / 5	1	1
C1	n.a.	n.a.	11.982	n.a.	n.a.	n.a.	0.630	3 / 2	1	3
C2	n.a.	n.a.	22.098	n.a.	n.a.	n.a.	1.855	3 / 6	3	1
C3	n.a.	n.a.	16.320	n.a.	n.a.	n.a.	0.690	2 / 2	0	1
C4	n.a.	n.a.	19.360	n.a.	n.a.	n.a.	0.900	4 / 4	n.a.	1
C5	n.a.	n.a.	16.035	n.a.	n.a.	n.a.	0.760	2 / 2	n.a.	3