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Glycomic Characterization of Prostate Specific Antigen and Prostatic Acid Phosphatase in Prostate Cancer and Benign Disease Seminal Plasma Fluids

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Abstract

Prostate-specific antigen (PSA) and prostatic acid phosphatase (PAP) are glycoproteins secreted by prostate epithelial cells, and have a long clinical history of use as serum biomarkers of prostate cancers. These two proteins are present at significantly higher concentrations in seminal plasma, making this proximal fluid of the prostate a good source for purifying enough protein for characterization of prostate disease associated changes in glycan structures. Using seminal fluid samples representative of normal control, benign prostatic disease and prostate cancers, PAP and PSA were enriched by thiophilic absorption chromatography. Released N-linked glycan constituents from both proteins were analyzed by a combination of normal phase HPLC and MALDI-TOF spectrometry. For PSA, 40 putative glycoforms were determined, and 21 glycoforms were determined for PAP. PAP glycans were further analyzed with a hybrid triple quadrupole/linear ion trap mass spectrometer to assign specific glycoform classes to each of the three N-linked sites. The glycans identified in these studies will allow for more defined targeting of prostate disease-specific changes for PAP, PSA and other secreted prostatic glycoproteins.

Keywords

prostate specific antigen; prostatic acid phosphatase; glycomics; mass spectrometry; biomarker

INTRODUCTION

The clinical measurement of the concentration and activities of two prostate derived glycoproteins, prostate specific antigen (PSA) and prostatic acid phosphatase (PAP), in serum has been assessed for decades in attempts to detect prostate cancers. Concentrations of PSA in serum above 10 ng/ml reliably indicate the presence of cancers, yet PSA testing is limited in its ability to differentiate prostate cancer from benign prostate hyperplasia (BPH), and specificity of the test diminishes significantly at lower PSA values below 10 ng/ml^{1–3}. Digital rectal exams (DRE) and determination of serum prostatic acid phosphatase (PAP) were the

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principal method for prostate cancer detection and staging before the advent of PSA testing, and they too have many limitations^{4, 5}. Serum PAP levels can still be used as an indicator of metastatic disease and possibly recurrence prediction^{6, 7}, but it has proven to have low specificities and sensitivities as a detection biomarker as compared to PSA⁸. While X-ray crystal structures have been reported for both human PSA and PAP^{9, 10}, there was little resolution of the glycan constituents of these proteins. Because of the known associations of changes in glycosylation associated with cancer progression^{11–14}, characterization of the glycan constituents of PSA and PAP derived from clinical samples representing healthy and different prostatic disease states could identify new biomarker candidates for prostate cancer detection.

PSA contains 8% carbohydrate by mass¹⁵, and has one glycosylation site at asparagine (Asn)-45¹⁶. Previous studies on PSA glycosylation have compared seminal plasma from healthy donors to prostate tumor metastatic cell line LNCaP^{17–19}. PSA purified from the seminal fluid of healthy subjects have been characterized as having sialylated complex biantennary glycans, frequent core fucose modifications, high mannose glycans, and some terminal N-acetylgalactosamines^{19, 20}. PSA glycans isolated from LNCaP cell lines have also been characterized as having some triantennary structures¹⁸, as well as decreased sialic acid content and the presence of increased fucose and GalNAc content¹⁷. A recent comparison of the sialic acid content of free PSA and proPSA isolated from serum, seminal plasma and tissues from non-cancer control subjects and prostate cancer subjects was reported²¹. Essentially no differences in sialylation content for the different forms of PSA were detected for any condition or sample²¹. A separate study of serum PSA derived glycans compared structural differences between free and complexed PSA²². Both forms of PSA were found to have mostly sialylated and fucosylated biantennary structures, while a few multiantennary complex structures were also identified²². These previous studies did not evaluate the specific structural configurations for each oligosaccharide isolated, and very few numbers of clinical samples were used. PAP has three glycosylation sites at Asn-62, Asn-188, and Asn-301^{9, 23}, making it a more difficult target for site specific structural characterization, yet one that may be a suitable complement to PSA. An X-ray crystal structure of PAP from normal seminal plasma had reported that Asn-301 had glycan modifications consisting of primarily high mannose oligosaccharides, while glycans at Asn-188 were less resolved, but consistent with complex structures containing sialic acid and fucose⁹. In relation to prostate cancer, a previous serial lectin analysis of PAP isolated from tissue in BPH and cancer subjects reported a decrease in high-mannose and hybrid glycans with core fucoses in cancer relative to BPH, and an increase of non-fucosylated hybrids in PCa as compared to BPH²³.

The objective of our study described herein was to obtain purified forms of both PSA and PAP from a clinical cohort of seminal plasma from healthy, BPH and prostate cancer subjects for further glycan structural analyses. We hypothesized that characterization of these PSA and PAP glycoforms across specified clinical sample sets would identify a subset of glycan constituents as biomarker candidates for the detection of prostate cancers. Following a purification step using thiophilic chromatography, the released glycans from PSA and PAP were characterized using HPLC separation of aminobenzoate derivatives, permethylation and MALDI-TOF/TOF profiling, and glycopeptide analysis on a hybrid triple quadrupole/linear ion trap mass spectrometer. The cumulative structures obtained for both PSA and PAP are reported and potential disease specific classes of glycoforms are identified. Specific assignments of glycan structural subtypes have also been determined for each of the three glycosylation sites on PAP. The glycan structures reported for PAP represent the first description of its glycan constituents using mass spectrometry based methods.

EXPERIMENTAL

Seminal plasma samples

Seminal fluid was obtained from men seen at the EVMS Department of Urology/Divine Tidewater Urology clinic for prostate cancer screening from 1989–2003. A semen collection kit was provided for each donor. Following semen collection, an instant freeze pack was applied to each tube for storage, and brought with the donor to the urology clinic. Following transport to the Virginia Prostate Center biorepository at EVMS, each sample was thawed, centrifuged to remove sperm, and stored in 0.5 ml aliquots. Initially, Normal (n=65), BPH (n=59), and PCa (n=92) pools (8–10 mls total/pool) were compiled from samples previously aliquoted for expression profiling studies (0.05 to 0.2 ml per sample), and matched for age. Each aliquot went through less than 2 freeze/thaw cycles, and average serum PSA values were 10.4 ng/ml (range 0.01 – 225.8) for PCa, 3.6 ng/ml (range 0.15 – 13.9) for BPH, and 1.5 ng/ml (range 0.3 – 4.8) for normal. The pooled samples were then subjected to a low-speed spin (4°C, 12k RPM, 20 minutes) to remove any cellular contaminants, followed by ultracentrifugation (4°C, 37K RPM, 1 hour) to pellet out the prostasomes. The supernatant was collected and 400ul aliquots were stored at –80°C. A more defined subset of seminal plasma pools were created for each clinical group, with each cohort having defined serum PSA values in the 2–7 ng/ml range. Nine samples, 500 µl per sample, were used to make each seminal plasma pool, and the samples used were collected between 1997 and 2003. The mean serum PSA values were 3.58 ng/ml for normal, 2.64 ng/ml for BPH, and 5.83 ng/ml for prostate cancer. BPH cases were defined as being biopsy negative, and the cancer samples were predominantly Gleason grade 6 (3+3). These pools were processed as previously described. The final supernatant of approximately 4.5 ml was aliquoted and stored at –80°C until needed. Protein concentrations were determined for all seminal plasma pools.

Thiophilic adsorption chromatography (TAC)

PSA and PAP were purified from seminal plasma pools using thiophilic adsorption chromatography (TAC) following the methods outlined by Kawiniski et al.²⁴. Briefly, seminal plasma pools were applied to Fractogel® EMD TA “T-gel” (Merck KGaG, Darmstadt, Germany) columns (2 cm × 0.5 cm) equilibrated in 1M sodium sulfate, and eluted in decreasing salt fractions. Protein content of each pool was normalized to 3.0 mg, with the samples in a final volume of 1ml in 25mM HEPES/1M sodium sulfate. Next, the sample was applied to the column, followed by 2ml of column buffer, and allowed to flow through by gravity; this three milliliter fraction collected is named “unbound”. The column was washed with 8 bed volumes of column buffer. Batch elution with decreasing sodium sulfate molarities (0.8M, 0.6M, 0.4M, 0.2M, and 0.0M) was performed using a low-pressure peristaltic pump. For each concentration, approximately 3ml fractions were collected, aliquoted, and stored at –80°C. The presence of PSA and PAP were confirmed by Western blot, and MALDI peptide mass fingerprinting of the corresponding excised gel bands was done to assess relative purity. MASCOT scores of greater than 100 (significance threshold of 54) and sequence coverage of greater than 45% were routinely obtained for both PAP and PSA bands.

Normal Phase HPLC

Rapid glycan sequencing of both PSA and PAP was performed by using previously optimized procedures²⁵. Briefly, PSA and PAP gel slices were digested with PNGase F, then the released free glycans were labeled with 2-aminobenzamide (2-AB) for subsequent normal phase HPLC analysis²⁶. A subset of samples were treated with *Arthrobacter ureafaciens* sialidase as previously described by Guile *et al.*²⁷. The resulting peaks, separated by time of appearance, correspond to specific glycan structures on the basis of glucose unit values (data not shown)²⁷. All HPLC analyses were performed using a Waters Alliance HPLC System and quantified using the Millennium Chromatography Manager (Waters Corporation, Milford, MA).

N-glycan permethylation

TAC-purified PSA and PAP gel bands derived from seminal plasma pools were permethylated²⁸ following trypsin digestion. Gel bands were first reduced and alkylated, and then dried *in vacuo* prior to proteolytic digestion. Trypsin digestions were performed at 37°C for 18 hours, followed by peptide extraction with 50% acetonitrile/0.1% TFA and dried *in vacuo*. Next, trypsin was denatured by heating the sample at 100°C for 5 minutes, and 1500 units of PNGase F (New England Biolabs, Ipswich, MA) was added to the peptide mixture and incubated at 37°C for 18 hours. The final digested product was purified using Resprep™ C18 cartridges (Restek Corporation, Bellefonte, PA). The N-glycan fraction was collected in 5% acetic acid, and subsequently dried under reduced pressure. Dried N-glycans were permethylated following the protocol described by Ciucanu and Kerek²⁸. The permethylated N-glycans were purified using C18 columns, eluted in 85% acetonitrile, and then dried under a nitrogen stream.

MALDI-TOF/TOF

The dried permethylated N-glycans were reconstituted in 20 µl of 100% methanol. Sample was mixed 1:1 with 2,5-Dihydroxybenzoic acid (DHB) matrix (20mg/ml in 50% methanol) and spotted on a polished steel MALDI-TOF target plate and allowed to crystallize at room temperature in the dark. Each sample was analyzed in positive ion mode using an UltraFlex III MALDI-TOF/TOF instrument (Bruker Daltonics, Germany). FlexControl and FlexAnalysis software (Bruker Daltonics, Germany) were used for spectra processing. Additionally, the glycan database offered by the Consortium for Functional Glycomics (<http://www.functionalglycomics.org>) was used to search permethylated glycan masses correlating to peaks of interest in MALDI-TOF spectra. Glycan “cartoons” representing mass peaks were built using GlycanBuilder ver 1.2 build 3353²⁹.

Hybrid triple quadrupole/linear ion trap mass spectrometry

A hybrid triple quadrupole/linear ion trap mass spectrometer 4000 (QTRAP® LC/MS/MS system, Applied Biosystems, Foster City, CA) coupled to a Tempo NanoLC system (Eksigent Technologies, Dublin, CA) was used to determine the structures of the glycans attached to each of the three PAP linkage sites: Asn-62, Asn-188, and Asn-301. The methods described by Sandra et al.³⁰ were used with minor modifications to optimize for our samples and instrumentation. Briefly, TAC-purified PAP gel slices derived from seminal plasma were reduced, alkylated, and digested with a saturated solution (in 50mM ammonium bicarbonate) of either 750 ng of trypsin or 950 ng of chymotrypsin. After an overnight digestion, the resultant peptides were extracted from the gel slice using 50% acetonitrile/0.1% TFA in water and dried *in vacuo*. The digested product was reconstituted in 20 µl of Buffer A (5% acetonitrile/0.1% formic acid/0.005% heptafluorobutyric acid in water) and 8 µl was injected into the nanoLC system for fractionation and analysis. Both the linear ion trap and the triple quadrupole capabilities of the QTRAP were utilized in these analyses. Glycopeptides were identified in the digestion mixtures by monitoring for unique marker oxonium ions such as m/z 163 (Hex⁺), 204 (HexNAc⁺), 292/274/256 (NeuAc⁺), and 366 (HexHexNAc⁺) that originate from fragmented glycopeptides^{30, 31}. In this approach the peptides are scanned in quadrupole 1 (Q1) to determine their masses and retention times. The peptides are then transmitted to quadrupole 2 (Q2) which acts as a collision cell where fragmentation occurs. Quadrupole 3 (Q3) is set to transmit only the mass of the diagnostic oxonium ions. Upon their detection, an enhanced product ion scan (EPI) of the precursor ion is triggered where fragmentation occurs in Q2, the fragmented ions are captured in the ion trap and then scanned out generating an MS/MS spectrum containing the ions from the both the peptide and the linked carbohydrate structures.

RESULTS

Purification of PSA and PAP from seminal plasma pools

A large repository of seminal plasma fluids collected over the last 18 years were used to create pooled sample cohorts representative of normal control, benign prostatic hyperplasia (BPH) and prostate cancer conditions. A thiophilic adsorption chromatography (TAC) approach adapted from Kawiniski et al.²⁴ was used to purify PSA and PAP from the prostasome depleted, pooled seminal plasma fluids. Following sequential batch elution, the resulting protein fractions were separated by SDS-PAGE, as shown in Figure 1. Under the conditions used, PAP did not bind to the TAC resin, and PSA eluted in both the 0.6M and 0.4M sodium sulfate fractions. Both PSA and PAP were readily detected by Coomassie staining, and their identities were confirmed in their respective fractions by western blotting and peptide mass fingerprinting. For both PSA and PAP isolated from gel slices, MASCOT scores of over 100 and sequence coverage of at least 45% were obtained. There was no indication of other protein sequences present with significant scores.

Rapid glycan sequencing analysis of PAP and PSA glycans

The TAC fractions containing PSA and PAP were used for preparative SDS-PAGE separations, stained with Coomassie blue, and gel slices corresponding to PSA or PAP excised. Each gel band was digested with trypsin, followed by PNGaseF digestion and 2-aminobenzamide derivatization of the released glycans as previously described²⁶. Glycans from PSA and PAP for each of the three clinical groups were then separated by normal phase HPLC and their elution positions used to define structural classes²⁷. The elution profile of PAP derived 2AB modified glycans from each pool is shown in Figure 2. It is clear that there are major differences in glycosylation patterns detected for PAP, particularly in the comparison of healthy and benign relative to the cancer samples. Because of the known instabilities of terminal sialic acid residues during processing and HPLC analysis²⁶, digestion with *Arthrobacter ureafaciens* sialidase was also done prior to normal phase HPLC separation to compare the non-sialylated structures. As summarized in Figure 3, there were still significant decreases in the fucosylated bi- and tetraantennary classes, FcA2G2 and FcA4G4, in the cancer samples. Either with or without sialidase treatment, there was also an apparent decrease in high mannose (Man6) structures in the cancer samples, and dramatic increases in cancer for truncated A1G1 structures. A similar analysis for 2-AB labeled PSA glycans was done, as summarized in Figure 4 (no sialidase) and Figure 5 (with sialidase). Cumulatively, the types of glycan structures detected in these HPLC separations are consistent with previously reported structures for PSA^{17–19}. There were no dramatic differences in expression of sub-types across the three clinical conditions, with the exception of the A2G2 structures in BPH in the non-sialidase treated preparation. Because this difference was not detected in the sialidase treated samples, this could reflect differential stabilities or sample processing differences. This will need to be further investigated in follow up studies.

The seminal plasma pools used for these initial analyses represent a broad range of clinical samples reflecting the disease severity spectrum of prostate cancers and benign conditions collected from 1990 to 2003, a time frame that spans the pre-PSA testing era to current clinical practice. Using the PSA levels for each individual in the pool of these samples, a refined pooled subset of 9 samples per condition was generated based on dates of collection after 1997 and serum PSA levels between 2–7 ng/ml. This PSA range better reflects the majority of benign and prostate cancer subjects detected in the PSA screening/testing era, and hence results from these samples should be more reflective of current urological practice. The same sample preparation and TAC purification was done for each new pool. PAP and PSA eluted in their respective fractions as described in Figure 1 (data not shown). As shown in Figure 6 for PAP, similar results to the larger pool data were obtained. There continued to be a larger proportion

of fragmented glycans (A1G1, FcA2, A2G1, FcA2) in the prostate cancer samples relative to the healthy and BPH cohorts. The results for PSA were essentially the same as shown in Figure 4 (data not shown). PSA and PAP purified from this new cohort was used for all subsequent analyses.

MALDI-TOF analysis of permethylated PSA and PAP glycans

In order to further define the structural repertoire of PSA and PAP glycans, permethylation²⁸ of the PNGaseF released glycans was done for each clinical cohort, followed by MALDI-TOF/TOF analysis. Because of the broad diversity of the indicated structural classes from the HPLC analyses, the goal was not to identify disease specific changes, but to better define all of the possible glycan structures detected on seminal plasma PSA and PAP. Permethylation of glycans aids in the stability of the terminal sialylated residues for detection by MALDI-TOF/TOF, and simplifies the spectra to primarily Na⁺ adducts, allowing for less complex annotation of mass peaks. From the permethylation experiments, we were able to detect multiple glycoforms for both PAP and PSA (Tables 1 and 2). A representative MALDI-TOF spectra of PAP derived permethylated glycans from prostate cancer samples are shown in Figure 7. Cumulatively, 21 structural classes of PAP glycans were detected representing primarily high-mannose and complex subtypes, with a few potential hybrid sub-types represented as well (Table 1). Permethylated glycans cleaved from PSA seemed to be mostly bi- and tri-antennary structures of the complex sub-type, but represented 40 potential classes, including high mannose and hybrid sub-types detected as well (Table 2). These results are consistent with structures previously reported for PSA glycans from serum and seminal fluids^{17–19, 21}.

Triple-quadrupole mass spectrometry analysis of PAP glycopeptides

Because PAP has three distinct glycan sites, and the previously reported crystal structure was only able to definitively assign one high-mannose containing site, individual glycopeptide analysis of the three sites was initiated. SDS-PAGE separated PAP was proteolytically digested with trypsin or chymotrypsin, and peptides fractionated by nanoLC system in-line with triple quadrupole mass spectrometer. The instrument was operated in the precursor ion (PI) scan mode for individual sugar molecules at specific m/z values of either 163 for Hexose⁺¹(Hex), 204 for N-acetylhexoseamine⁺¹ (HexNAc), 292/274/256 for N-acetylneuraminic acid⁺¹ (NeuAc), or 366 for Hex-HexNAc⁺¹. An enhanced product ion scan is triggered upon detection of the specified diagnostic ion, generating information on the sugar composition and the amino acid sequence of the glycopeptides in a single assay. An example is shown in Figure 8 for the tryptic peptide with one missed cleavage encompassing the high mannose site at Asn 301 (GEYFVEMY^RNETQHEPYPLMLPGCSPSCPLER), as determined by the precursor ion scan for oxonium ion 163. Knowing the parent mass allows for annotation of the remaining triply charged ion peaks in the third quadrupole (Q3) for identification of sugar constituents and fragments. The annotation of these glycans was greatly simplified as this site contains only high mannose structures, with Man6 being the major constituent, and Man7 a minor one, which is consistent with the PAP crystal structure⁹. As listed in Table 3, this approach has been useful for identifying complex biantennary and triantennary glycans at Asn 62. This site had previously been poorly resolved in the PAP crystal structure, such that complex glycan structures were proposed to be unlikely constituents⁹. Like in the crystal structure, the site at Asn 188 has proven elusive to glycan analysis, particularly when trypsin was used for digestion, which we believe was due to a combination of missed tryptic cleavages combined with large tetra-antennary glycans. However, use of chymotrypsin allowed detection of Asn 188 containing glycopeptides, consistent with attached tetraantennary glycan structures (Table 3).

DISCUSSION

The need for improved prostate cancer biomarkers beyond the serum PSA test is increasingly evident. Because this test is applied to population screening, there is a wealth of data that indicates its strength in increasing detection of prostate cancers, but its use has also led to increased over-treatment and unnecessary surgeries for indolent disease. Despite its widespread use, there is still much characterization of PSA to be done, particularly in regards to its structural glycosylation properties and physiological function. Proximal fluids of the prostate like seminal fluid that are enriched for prostate derived secreted glycoproteins like PSA have long been a source of this enzyme, particularly as it is generally present at concentrations that range from 100–400 µg/ml in this fluid. Using a large cohort of archived seminal plasma samples reflective of healthy, benign and prostate cancer conditions allowed systematic characterization of PSA for similarities and differences in its disease specific glycosylation patterns. While PSA glycosylation has been previously studied by many other groups^{17–19, 21, 32}, to our knowledge we are the first to examine PSA glycoforms across matched, disease-defined sample sets reflective of multiple samples. For a glycoprotein with one site of N-glycosylation, the number of different glycan structures identified across the conditions analyzed is highly variable, ranging from predominant complex bi- or tetraantennary structures, to hybrid and high mannose glycoforms. In this regard, use of pooled samples to identify disease specific variants of PSA will be highly limiting. While it is appropriate to define the repertoire of potential structures, there were no conclusions that could be reached regarding specific disease glycoform variants of PSA. Methods that facilitate characterization of individual samples like targeted lectin-ELISA assays³³ or specific selective reaction monitoring (SRM) approaches using instrumental configurations like the hybrid triple quadrupole MS assay for PAP will be necessary to better define disease specific glycoform changes for PSA. The diversity of glycan structures detected for PSA, and the well established role of PSA in clinical medicine and prostate disease, certainly warrant these types of approaches on larger sample cohorts.

The cumulative glycan results for PAP represent the first characterization of PAP structures using mass spectrometry approaches. Previous reports indicated that PAP glycosylation at Asn-301 was of the high-mannose type, while it was hypothesized that the Asn-188 site contained complex-type glycans and the Asn-62 site contained non-complex structures⁹. Our results confirm that high-mannose glycoforms are present at the Asn-301 site, with the presence of Man6 and Man7 glycans observed for this site. Based on the data summarized in Table 3, we have found that sialylated complex-type glycans occupy the site at Asn-62, with bi- and tri-antennary structures present. The glycans attached to Asn-188 are less defined, but the preliminary results indicate a larger glycan constituent, most likely the tetra-antennary complex sialylated and fucosylated structures observed in the characterization studies (Table 2). Clearly, application of alternative proteases besides trypsin could better clarify the glycopeptide constituents of the Asn-188 site. Emerging improvements in computer programs like SimGlycan (Applied Biosystems/PremierBiosoft) that allow annotation of large complex glycan constituents will also facilitate this characterization. Conversely, the HPLC and MALDI-TOF profiling indicated the presence of truncated glycan species predominantly in the prostate cancer samples. Their further characterization at the structural level, as well as their site of attachment, could lead to specific PAP glycan biomarker assays. Additionally, the described purification and analysis approaches for both PAP and PSA glycans are compatible with emerging quantitative isotope labeling strategies³⁴. Missing for both PAP and PSA are any determinations of the anomeric linkages for the different glycan species, as linkage differences and positions of the sialic acid residues in particular could dictate biological and clinical differences. The permethylation strategies already utilized are equally valid for providing PSA and PAP glycans for more detailed MS/MSⁿ structural characterization approaches^{35, 36}, and these are currently ongoing in our laboratories.

The cumulative glycan structural information from PSA and PAP is being used in a prostate cancer biomarker “pipeline” to develop specific lectin-ELISA assays³³ targeting fucosylation and sialylation differences, as well as targeted SRM and/or multiple reaction monitoring (MRM) assays in proximal prostatic fluids. These approaches are particularly advantageous for the analysis of many individual seminal plasma samples, as well as other proximal prostatic fluids termed expressed prostatic secretions (EPS) being collected for molecular genetic biomarker assays^{37, 38}. Expressed prostatic secretions are collected in voided urine following a standard urological digital rectal exam combined with prostate massage, and analysis of their glycoprotein constituents could complement and extend results reported in seminal plasma. In conclusion, there are multiple glycan targets of potential clinical relevance that warrant continued structural analysis of PAP and PSA glycopeptide species. The use of seminal plasma derived proteins will continue to facilitate this characterization, and can also be readily adapted to other clinical relevant proximal fluids of the prostate.

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Figure 1. Seminal plasma fractionation by thiophilic adsorption chromatography

Seminal plasma protein fractions purified by thiophilic adsorption chromatography (TAC) were separated on a 12% Bis-Tris NuPAGE SDS-gel (Invitrogen) and stained with Coomassies blue. Unbound proteins are shown in Lane 1, with protein from the 0.8M NaSO₄ elution in Lane 2, 0.6M (Lane 3), 0.4M (Lane 4), 0.2M (Lane 5) and 0 M/25mM HEPES (Lane 6). This gel shows the proteins from normal seminal plasma, and is representative of the purifications of the benign and cancer samples. PAP and PSA identities were confirmed in the above fractions by staining, western blots and MALDI-TOF analysis.

Figure 2. Normal phase HPLC separation of 2-AB derivatized N-linked glycans from PAP
 The relative levels of N-linked glycans from PAP isolated from normal control, benign (BPH) and prostate cancer (PCa) conditions separated by normal phase HPLC. Structural designation are as follows: A1G1, hybrid glycan with a single galactose residue on the 1,6 arm; FcA2, core fucosylated (1,6) agalactosylated biantennary glycan; Man6, High mannose glycan with 6 mannose residues, A2G2, biantennary N-glycan; Man7, High mannose glycan with 7 mannose residues; A3G3, triantennary N-linked glycan; FcA3G3, core fucosylated (1,6) triantennary N-linked glycan; A4G3, tetraantennary N-linked glycan with 3 galactose residues; A4G4, tetraantennary N-linked glycan; FcA4G4, core fucosylated (1,6) tetraantennary N-linked glycan. B indicates bisecting GlcNAc residues.

Figure 3. Normal phase HPLC separation of 2-AB derivatized N-linked glycans from PAP treated with sialidase

N-linked glycans from PAP isolated from normal control, benign (BPH) and prostate cancer (PCa) were treated with sialidase prior to normal phase HPLC separation. Shown are the relative intensities of each indicated structure per condition, normal (Gray bars); BPH (Black bars); PCa (White bars). Structural abbreviations are described in the legend for Figure 2.

Figure 4. Normal phase HPLC separation of 2-AB derivatized N-linked glycans from PSA
N-linked glycans from PSA isolated from normal control, benign (BPH) and prostate cancer (PCa) were separated by normal phase HPLC. Shown are the relative intensities of each indicated structure per condition, normal (Gray bars); BPH (Black bars); PCa (White bars). Structural abbreviations are described in the legend for Figure 2

Figure 5. Normal phase HPLC separation of 2-AB derivatized N-linked glycans from PSA treated with sialidase

N-linked glycans from PSA isolated from normal control, benign (BPH) and prostate cancer (PCa) were treated with sialidase prior to normal phase HPLC separation. Shown are the relative intensities of each indicated structure per condition, normal (Gray bars); BPH (Black bars); PCa (White bars). Structural abbreviations are described in the legend for Figure 2

Figure 6. Normal phase HPLC separation of 2-AB derivatized N-linked glycans from PAP from the refined second sample pool

N-linked glycans from PAP isolated from the second pool of normal control, benign (BPH) and prostate cancer (PCa) seminal fluids stratified to PSA levels and other clinical parameters. Shown are the relative intensities of each indicated structure per condition following normal phase HPLC separation: normal (Gray bars); BPH (Black bars); PCa (White bars). Structural abbreviations are described in the legend for Figure 2

Figure 7. MALDI-TOF analysis of permethylated PAP glycans from prostate cancer seminal plasma

A representative MALDI spectra for permethylated PAP is shown. Combined with the structures determined in the control and BPH permethylated PAP samples, glycan structures corresponding to detected m/z peaks are listed in Table 1.

Figure 8. Precursor ion scan for 163 m/z hexose of tryptic PAP peptides

A) Enhanced Product Ion (EPI) scan of a quadrupole-charged PAP glycopeptide with parent mass of 5449.0. B) Expanded window of above EPI scan showing fragmentation of the parent ion that corresponds to the tryptic peptide

GEYFVEMYRNETQHEPYPLMLPGCSPSCPLER containing the glycosylation site Asn-301 and a Man6 N-glycan.

Table 1**Permethylated MALDI-TOF m/z and corresponding N-glycan structures for PAP**

Permethylated PAP glycans from normal, BPH, and PCa seminal plasma pools were analyzed by MALDI-TOF. Identified m/z values were used for database searches, and corresponding masses that could be assigned to a biologically relevant N-glycan structure are listed. An asterisk denotes structures that are one representation of multiple possible glycoforms for a given mass. The shown structure in these cases was chosen based on its apparent prevalence in mammalian species. Cartoon representations are as follows: ■=GlcNAc, ●=Mannose, ●=Galactose, ▲=Fucose, ◆=NeuAc, ■=GalNAc

Number	m/z	Proposed Glycan Structure	Sub-Type
1	1784.016	M6N2 	High Mannose
2	1987.958	M7N2 	High Mannose
3	2070.189	Gal2N2M3N2 	Complex
4	2244.268	Gal2N2M3N2F 	Complex
5	2314.091	Hex5HexNAc5 	Complex
6	2432.339	NeuAc1Gal2N2M3N2 	Complex
7	2606.425	NeuAc1Gal2N2M3N2 	Complex
8	2793.503	NeuAc2Gal2N2M3N2 	Complex
9	2881.559	NeuAc1Gal3N3M3N2 	Complex
10	2967.587	NeuAc2Gal2N2M3N2F 	Complex
11	3055.647	NeuAc1Gal3N3M3N2F 	Complex
12	3242.719	NeuAc2Gal3N3M3N2 	Complex
13	3416.793	NeuAc2Gal3N3M3N2F 	Complex
14	3504.838	NeuAc1Gal4N4M3N2F 	Complex
15	3603.894	NeuAc3Gal3N3M3N2 	Complex

Number	m/z	Proposed Glycan Structure	Sub-Type
16	3777.971	NeuAc3Gal3N3M3N2F 	Complex
17	3867.027	NeuAc2Gal4N4M3N2F x2 	Complex
18	3963.779	Hex6HexNAc5NeuAc4 	Complex
19	4053.873	NeuAc3Gal4N4M3N2 x3 	Complex
20	4227.294	NeuAc3Gal4N4M3N2F x3 	Complex
21	4588.57	NeuAc4Gal4N4M3N2F 	Complex

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Number	m/z	Proposed Glycan Structure	Sub-Type	Number	m/z	Proposed Glycan Structure	Sub-Type
11	2419.445	Hex5dHex2HexNAc4	Complex	31	3242.891	NeuAc2Gal3N3M3N2	Complex
12	2432.413	NeuAc1Gal2N2M3N2	Complex	32	3345.936	Hex8dHex1HexNAc5	Complex
13	2472.384	NeuAc1GalN3M3N2	Complex	33	3416.859	NeuAc2Gal3N3M3N2F	Complex
14	2518.443	Gal3N3M3N2	Complex	34	3593.05	Hex8dHex1HexNAc7	Complex
15	2549.473	Hex6dHex3HexNAc3	Hybrid	35	3605.045	NeuAc3Gal3N3M3N2	Complex
16	2561.577	Hex5HexNAc6	Complex	36	3777.062	NeuAc3Gal3N3M3N2F	Complex
17	2593.532	Hex5dHex3HexNAc4	Complex	37	3866.523	NeuAc2Gal4N4M3N2F	Complex
18	2606.502	NeuAc1Gal2N2M3N2F	Complex	38	3912.098	Hex9dHex1HexNAc6NeuAc	Complex
19	2677.545	NeuAc1Gal2N3M3N2	Complex	39	3965.198	Hex6HexNAc5NeuAc4	Complex
20	2694.765	Gal3N3M3N2F	Complex	40	4416.599	NeuAc4Gal4N4M3N2	Complex

Table 3**PAP glycopeptides with corresponding N-glycan structures identified by QTRAP**

Both tryptic and chymotryptic PAP digests were analyzed by the QTRAP as described in the experimental section. PI scans for oxonium ions 163, 366, and 292 are represented in the above table. Certain EPI scans revealed glycopeptide information that allowed for the assignment of both peptide and glycan

N-linked Glycosylation site	Peptides generated by enzymatic digestion	Structures Identified
Asn-62	Trypsin Digestion FLNESYKHEQVYIR 1825.9 KFLNESYKHEQVYIR 1954.0
	Chymotrypsin Digestion RKFLNESYKHEQVY 1840.9	
Asn-188	Trypsin Digestion N/A
	Chymotrypsin Digestion CESVHNFTLPSW 1476.7 DPLYCESVHNFTLPSW 1964.9 YCESVHNFTLPSWATEDM 2304.0	
Asn-301	Trypsin Digestion NETQHEPYPLMLPGCSPSCPLER 2712.2 GEYFVEMYRNETQHEPYPLMLPGCSPSCPLER 4049.9
	Chymotrypsin Digestion RNETQHEPYPLM 1530.7 RNETQHEPYPLMLPGCSPSCPLERF 2974.3	