Glycobiology vol. 18 no. 1 pp. 2–8, 2008
doi:10.1093/glycob/cwm117
Advance Access publication on October 23, 2007

Oligosaccharide profiles of the prostate specific antigen in free and complexed forms from the prostate cancer patient serum and in seminal plasma: a glycopeptide approach

Michiko Tajiri[2,3], Chikara Ohyama[4], and Yoshinao Wada[1,2]

[2]Department of Molecular Medicine, Osaka Medical Center and Research Institute for Maternal and Child Health, 840 Murodo-cho Izumi, Osaka 594-1101, Japan; [3]CREST, Japan Science and Technology Agency, 4-1-8 Honcho Kawaguchi. Saitama 332-0012, Japan; and [4]Department of Urology, Hirosaki University School of Medicine, Hirosaki, Aomori 036-8563, Japan

Received on July 18, 2007; revised on October 16, 2007; accepted on October 16, 2007

The oligosaccharide structures of prostate specific antigen (PSA) are expected to be useful in discriminating prostate cancer from benign conditions both accompanied by increased serum PSA levels. A large proportion of PSA forms a covalent complex with a glycoprotein, α_1-antichymotrypsin, in human blood. In the present study, the glycan profiles of free and complexed forms of PSA from cancer patient serum and of seminal plasma PSA were compared by analyzing the glycopeptides obtained by lysylendopeptidase digestion of the electrophoretically separated PSA with mass spectrometry. The profiles of the PSA *N*-glycans from the free and complexed molecules were quite similar to each other and consisted of fucosylated biantennary oligosaccharides as the major class. They were mostly sialylated, and a considerable sialic acid fraction was α2,3-linked as determined by *Streptococcus pneumoniae* neuraminidase digestion of the glycopeptides. In the seminal plasma PSA, high-mannose and hybrid types of oligosaccharides were predominant, and the sialic acids attached to the latter as well as to biantennary oligosaccahrides were exclusively α2,6-linked because they were removed by *Arthrobacter ureafaciens* neuraminidase but resistant to *S. pneumoniae* neuraminidase. Complex-type oligosaccharides from other sources were found in the seminal plasma sample, indicating that analysis of released glycans carries a risk of being misleading. The results suggest that identification of α2,3-linked sialic acids on PSA potentially discriminates malignant from benign conditions, if the analysis is applied to oligosaccharides specifically attached to the *N*-glycosylation site of PSA in either a free or a complexed form in the serum.

Keywords: *N*-glycans/prostate specific antigen (PSA)/ prostate cancer/sialic acid

Introduction

Prostate cancer is one of the common cancers, and the incidence continues to rise in most regions of the world. The measurement of prostate specific antigen (PSA), which has been available for 20 years and led to the early detection, management and follow-up of patients with prostate cancer (Stamey et al. 1987), is considered to be one of the best biochemical markers currently available in the field of oncology. PSA is a serine protease belonging to the kallikrein multigene family and consists of 237 amino acids and one N-linked oligosaccharide chain at Asn45 (van Halbeek et al. 1985; Bélanger et al. 1995). The majority of PSA produced by the prostate is excreted in semen but a small proportion leaks into the systemic circulation. PSA testing is based on the fact that prostate cancer tissues release 30 times more PSA into the circulation than the normal prostate tissue, perhaps due to the loss of normal tissue architecture (Stamey et al. 1987). The PSA that reaches the serum is found either in free form or bound with plasma proteins. The major binding proteins are α_1-antichymotrypsin (ACT) (Christensson et al. 1990) and α_2-macroglobulin, both of which are extracellular protease inhibitors abundantly present in the serum. Complex formation with α_1-antichymotrypsin–prostate specific antigen (ACT–PSA) results in the exposure of a limited number of the antigenic epitopes of PSA, whereas α_2-macroglobulin encapsulates the currently identifiable antigenic epitopes of PSA (Christensson et al. 1990). ACT–PSA is, therefore, the predominant immunoreactive form in the serum, whereas free PSA accounts for 5–40% of total immunoreactive PSA (Jain et al. 2002). Measuring free and complexed PSA and determining their ratio improve the diagnostic specificity of PSA testing (Lilja et al. 1991; Stenman et al. 1991) and can decrease the number of negative prostatic biopsies by 20–25% (Catalona et al. 1998). However, the incidence of prostate cancer has been shown to be as high as 22% in patients with a normal PSA range of 2.6–4.0 ng/mL (Catalona et al. 1997). Furthermore, while the PSA test is essentially organ specific, it is not cancer specific with elevated serum concentrations found in noncancerous diseases such as benign prostatic hypertrophy and prostatitis. There has, therefore, been an increasing emphasis on the need for novel serum markers for use in the diagnosis of prostate cancer.

The carbohydrate structure of cancer cells is well known to differ considerably from that of nonmalignant cells (Fukuda 1996; Kim and Varki 1997), and a number of carbohydrate tumor markers have been used in clinical situations. Accordingly, the carbohydrate structures of PSA potentially discriminate cancer from benign diseases despite both showing serum PSA elevations. The structure of PSA carbohydrate is thought to be a biantennary complex-type oligosaccharide (Bélanger et al. 1995; Sumi et al. 1999; Prakash and Robbins 2000; Okada et al. 2001; Peracaula et al. 2003; Ohyama et al. 2004). PSA from prostate cancer tissues and a prostate cancer cell line was reported to contain the complex-type oligosaccharides with more antennas than the PSA from benign prostatic hypertrophy

[1]To whom correspondence should be addressed: Tel: +81-725-56-1220; Fax: +81-725-57-3021; e-mail: waday@mch.pref.osaka.jp

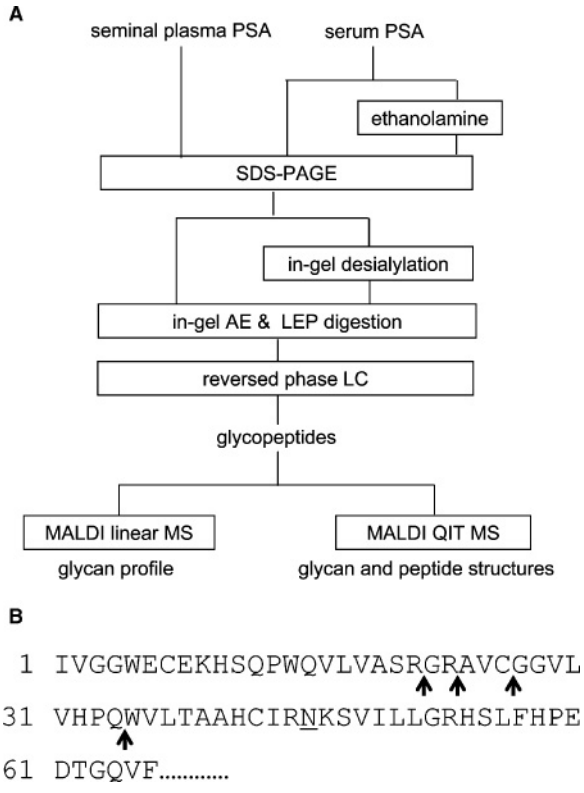
tissues and seminal fluid (Sumi et al. 1999; Prakash and Robbins 2000). Although an obvious extension of these studies is to characterize the oligosaccharides of the serum PSA, the low PSA content of the human serum makes this difficult, and only a few reports are available on the structural details of the PSA glycans from cancer patient sera (Ohyama et al. 2004; Tabarés et al. 2006). To date, most of the studies on PSA glycans have been carried out on the oligosaccharides released from PSA samples, and they are not inherently free of contaminating glycans from other sources. In the present study, on the other hand, glycopeptides were analyzed by mass spectrometry (MS). MS and tandem MS of glycopeptides allow characterization of the site-specific glycans of glycoproteins in an efficient and quite reliable manner (Wada et al. 2004; Tajiri et al. 2005). The glycan profiles specific to PSA were analyzed for the free PSA and ACT–PSA, in which ACT is also a glycoprotein, from two cancer patient sera as well as seminal plasma PSA and the differences with respect to fucosylation, sialylation, and sialic acid linkage were addressed.

Results and discussion

Seminal plasma PSA

The seminal plasma PSA has been used as an alternative to the normal PSA sample, since the serum PSA from individuals with normal prostates or benign prostatic hypertrophy is difficult to analyze due to inadequate sample amounts. However, the oligosaccharide structures of the seminal plasma PSA have been inconsistent among reports. An earlier report on nuclear magnetic resonance spectroscopy and electrospray ionization MS proposed a simple profile consisted of disialylated biantennary oligosaccharides with a core fucose (Bélanger et al. 1995), and a similar result was obtained by in-gel PNGase F digestion of the PSA band in sodiumdodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Prakash and Robbins 2000; Peracaula et al. 2003). On the other hand, a more diverse profile including a hybrid-type oligosaccharide has been reported (Ohyama et al. 2004).

To perform a specific profiling of the oligosaccharides at the *N*-glycosylation site of PSA, the tetrapeptide IRNK (positions 43–46) was obtained by in-gel S-aminoethylation of the proteins separated by SDS–PAGE (supplementary Figure 1) followed by lysylendopeptidase (LEP; *Achromobacter* protease I) digestion, and the resulting IRNK glycopeptide was then purified by reversed phase chromatography using a C30 column (Scheme 1A). Originally, we performed S-carbamidomethylation to obtain a longer peptide but the differently truncated N-terminal of PSA made the mass spectra complicated (see Scheme 1B). The MALDI linear TOF mass spectrum of the IRNK glycopeptide showed a considerable heterogeneity represented mainly by the hybrid-type oligosaccharides and by high-mannose and complex types as well (Figure 1A). The presence of these glycans was verified by an analysis of glycopeptides bound to Con-A (supplementary Figure 2). The complex-type oligosaccharides were fucosylated, but this modification involved half of the total glycans when estimated from their signal intensities, since hybrid and high-mannose types of oligosaccharides were barely fucosylated. The ion at *m*/*z* 2632.8 corresponded to the glycopeptide bearing a GalNAc–GlcNAc outer chain (Okada et al. 2001; Peracaula et al. 2003), but further analysis to differentiate the isomers was not carried out in the present study. The glycan structures, including core fucosylation, as well as the peptide backbone sequence were then verified by MALDI quadrupole ion-trap (QIT) tandem MS as described previously (Wada et al. 2004) (supplementary Figure 3). Interestingly, in this analysis, the glycopeptides derived from other proteins, e.g. Niemann-Pick C2 protein bearing a biantennary complex-type oligosaccharide, were found in the commercially available specimen. Recent peptidome studies have demonstrated that protein fragments circulate abundantly in blood (Villanueva et al. 2006). Considering that some noncovalently linked complexes as well as the reduction-resistant ester linkage do not dissociate in the presence of SDS (Stratikos and Gettins 1997), the in-gel PNGase F digestion after SDS–PAGE is not presumably free from the risk of contamination by oligosaccharides or small glycopeptides from other molecular sources. This pitfall can be eliminated by MS of glycopeptides.



Scheme 1. Glycan profiling of PSA and the N-terminal sequence of PSA. (**A**) Strategies for glycopeptide analysis. All the experiments were repeated at least twice. AE, S-aminoethylation; LEP, lysylendopeptidase. (**B**) Arrows indicate the truncation position in seminal plasma PSA. The glycosylation site, Asn45, is underlined.

PSA glycans from cancer patient sera

PSA forms a complex with an equimolar parner ACT via an ester bond in the serum (Peter et al. 2000), and ACT is a glycoprotein containing bi-and tri-antennary oligosaccharides at six *N*-glycosylation sites (Laine et al. 1991). Whether the PSA oligosaccharides in the free PSA form and in ACT–PSA have the same glycan structures is unknown. To address this issue and to characterize the oligosaccharide structures, the

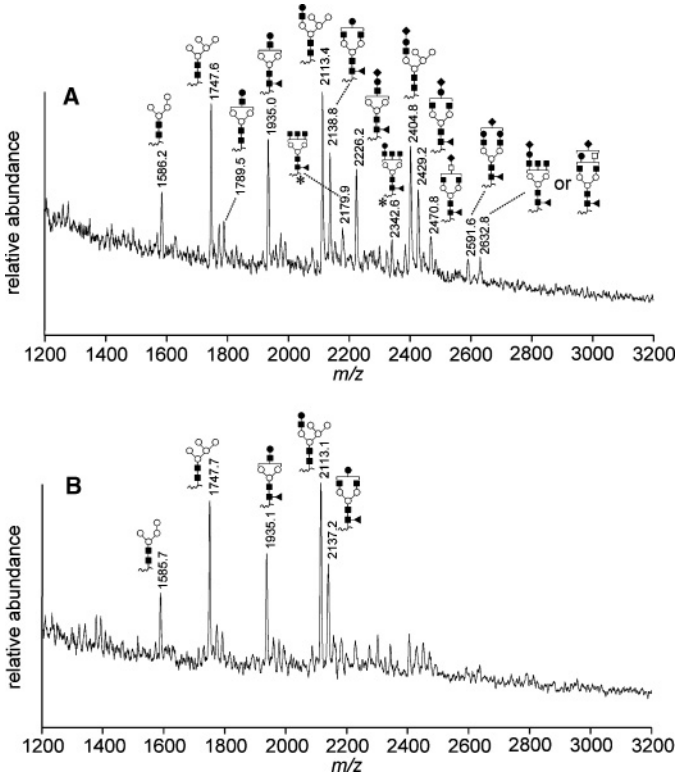


Fig. 1. MALDI mass spectra of the IRNK glycopeptide from seminal plasma PSA. Mass spectra of the glycopeptide bearing an IRNK peptide sequence (*Mr*529.7) from seminal plasma PSA before (**A**) and after treatment with *Arthrobacter ureafaciens* neuraminidase (**B**) were taken in a MALDI linear TOF mode. The values are average masses. The hypothetical glycan structures illustrated were deduced from their observed masses, and their glycosidic, cross-ring fragmentation patterns derived from tandem MS. They were also based on the structures reported in previous studies. For the structures marked with asterisks, one of the GlcNAc residues binding to the nonreducing end of mannose may be GalNAc linked to the nonreducing end of a GlcNAc as illustrated for the ion at *m*/*z* 2632.8 (see the text).

Table I. Fucosylated or sialylated glycopeptide contents

	Seminal plasma	Patient 1	Patient 2
Fucosylation	52%	100%	64%
Sialylation	31%	93%	88%
Sp Nase (Δ)	31% (−0%)	84% (−9%)	42% (−46%)
Au Nase (Δ)	9% (−22%)		

The contents were deduced from the relative abundances of their specific glycopeptide ions among total glycopeptide ions observed in the mass spectra shown in Figures 1 and 2. Mono-, di-, tri-, and tetra-sialylated species were not separately counted instead being included in the "sialylation" group. Nase, neuraminidase treatment; *Sp, Streptococcus pneumoniae; Au, Arthrobacter ureafaciens*. Δ, % change of sialylated species in total glycopeptides by neuraminidase treatment.

IRNK glycopeptide was obtained from either band (95 kDa for ACT–PSA and 30–32 kDa for free PSA in supplementary Figure 1) by in-gel LEP digestion. In both patients, the glycan profile of PSA was quite similar for ACT–PSA and free PSA, and was primarily consisted of biantennary oligosaccharides (Figure 2). The most abundant species was the fucosylated/sialylated one, while the relative abundances of the ions for fucosylated species among the total glycopeptide ions differed, 100% versus 64%, between these patients (Table I). In both patients, a small amount of the complex-type oligosaccharides with more antennas was identified. The present study confirmed, for the first time, that the oligosaccharides of the free and complexed forms of PSA are the same (Figure 2).

The ester bond between PSA and ACT was cleaved with alkaline ethanolamine treatment (Peter et al. 2000) (supplementary Figure 1). The glycan profile derived from thus increased "free" PSA was the same as that before treatment in both patients (data not shown).

Sialic acid linkage

The free PSA from the serum, prostate tissues, and seminal plasma forms multiple spots on two-dimensional electrophoresis. This was ascribed to sialylation and to the presence of the proPSA isoform, or the variability of the protein backbone (Tabarés et al. 2006, 2007), and the sialylation as well as fucosylation of PSA was expected to discriminate between benign and malignant prostate conditions (Peracaula et al. 2003). Furthermore, sialic acid linkage was expected to discriminate cancer from benign prostatic diseases in a study using lectin-affinity chromatography (Ohyama et al. 2004). In a study of released glycans, there was a decrease in α2,3-linked sialic acid in the serum PSA from prostate cancer patients compared with seminal plasma PSA (Tabarés et al. 2006). On the contrary, a study using lectin identified α2,3-linked sialic acids on the serum PSA from cancer patients but not on this from benign prostatic hypertrophy patients (Ohyama et al. 2004). In this study, the discrimination was more significant in free PSA than in the complexed form (mainly ACT–PSA) probably because the abundant oligosaccharides on ACT would obscure results specific to PSA.

In the present study, the PSA separated by SDS–PAGE was in-gel digested with *Streptococcus pneumoniae* neuraminidase, which removes only α2,3-linked sialic acid residues. Seminal plasma PSA, in which most sialylated glycans were of the hybrid-type, was not changed by the α2,3-neuraminidase digestion, while being disialylated by *Arthrobacter ureafaciens* neuraminidase (Figure 1B). By contrast, in the MALDI linear TOF mass spectrum, the ion at *m*/*z* 2301.0 in patient 1 and those at *m*/*z* 2154.1 and *m*/*z* 2300.4 in patient 2, all corresponding to the disialylated oligosaccharides were significantly increased (Figure 2C and F). Estimation from the signal intensity of ACT–PSA indicated that the α2,3-neuraminidase digestion changed 9% and 46% of the glycopeptides into the corresponding asialo species, in patients 1 and 2, respectively, while approximately 90% of glycopeptides were sialylated before treatment (Table I). Although the change was not drastic in patient 1, the relative abundance of the monosialylated species at *m*/*z* 2591.5 against the disialylated species at *m*/*z* 2882.8 was increased by this α2,3-neuraminidase treatment (Figure 2C). The sialic acid linkage thus determined was quite similar for free PSA and ACT–PSA in the same patient (data not shown).

The ionization of glycopeptides is basically dependent on protonation of the peptide backbone, and the signal intensity in the MALDI linear TOF mass spectrum approximately represents the relative quantity of the component glycans, while the minor extent of sialic acid loss is attributable to the prompt fragmentation before ion acceleration (Wada et al. 2007). In the present study, repeated analysis of the PSA from the patient serum and seminal plasma yielded quite reproducible mass spectra, justifying the glycan profile including the levels

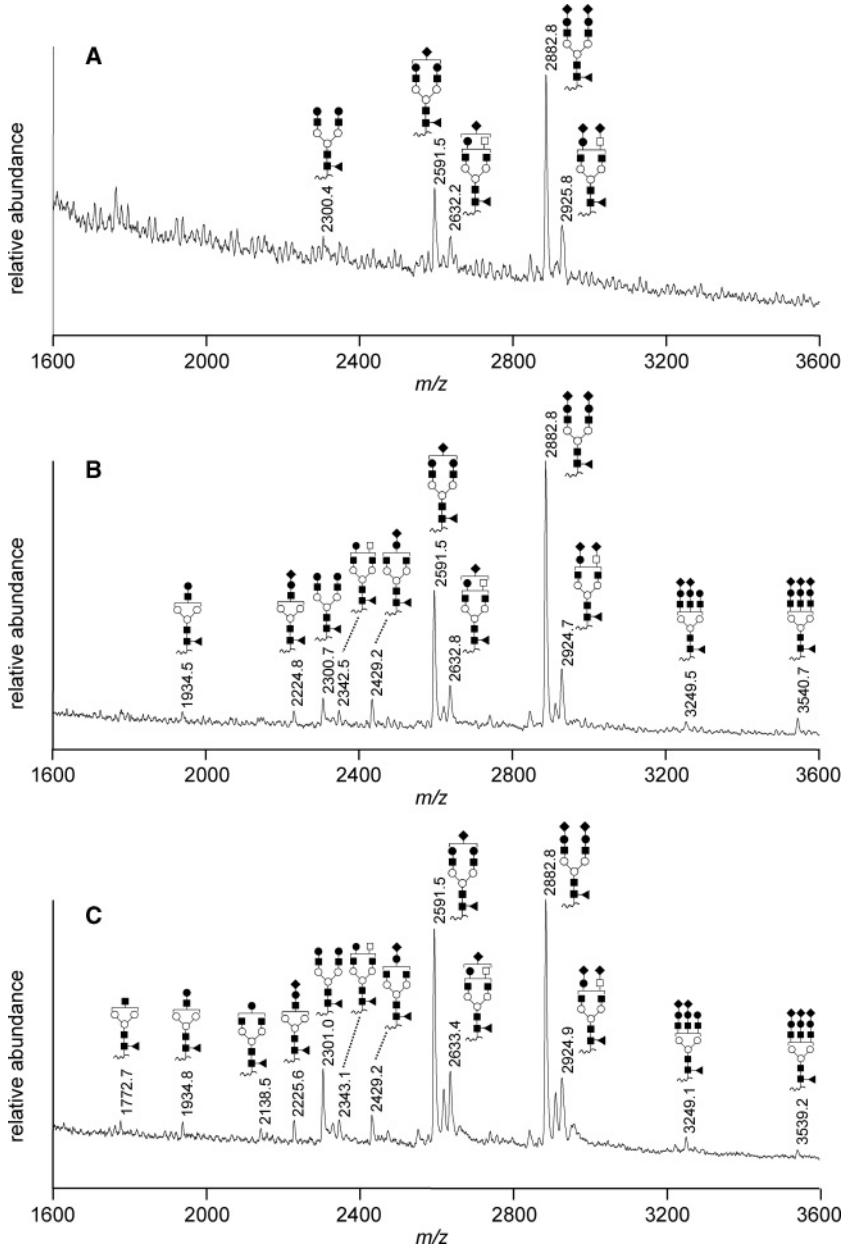


Fig. 2. Analysis of the IRNK glycopeptides from prostate cancer patient sera by MALDI MS. MALDI linear TOF mass spectra of the glycopeptides derived from free PSA (**A** and **D**) and ACT–PSA (**B** and **E**), and after digestion with *Streptococcus pneumoniae* neuraminidase (**C** and **F**). Patient 1 (**A**, **B**, and **C**); Patient 2 (**D**, **E**, and **F**). The values are average masses.

of sialylation, fucosylation, and sialic acid linkage thus obtained for PSA. Although the number of patients analyzed was small, our results suggest that the identification of α2,3-linked sialic acids potentially discriminates malignant from benign conditions, if the analysis is appropriately performed focusing on the oligosaccharides on the PSA molecule, in either free or complexed form, in the serum.

Materials and methods

Purification of PSA from the cancer patient serum

Prostate cancer sera were obtained from two patients whose serum PSA levels were 13,000 and 18,000 ng/mL for patients 1 and 2, respectively. Informed consent was obtained from both patients. Anti-PSA IgG (3 mg, ploYclonal) (Dako, Glostrup, Denmark) was coupled to a HiTrap NHS-activated HP column (1 mL bed volume, GE Healthcare, Fairfield, CT) according to the manufacturer's instructions. Sera (0.3–0.5 mL) were 10× diluted with PBS before sample loading, and the bound PSA was eluted with 0.1% trifluoroacetic acid. After being dried with a vacuum centrifuge, the samples were electrophoresed on a 10% SDS–polyacrylamide gel under reducing conditions. Seminal plasma PSA was purchased from Sigma (St. Louis, MO).

In-gel digestion

In-gel digestion was carried out according to the standard protocol (Shevchenko et al. 2006) except for the use of S-aminoethylation instead of S-carbamidomethylation. The

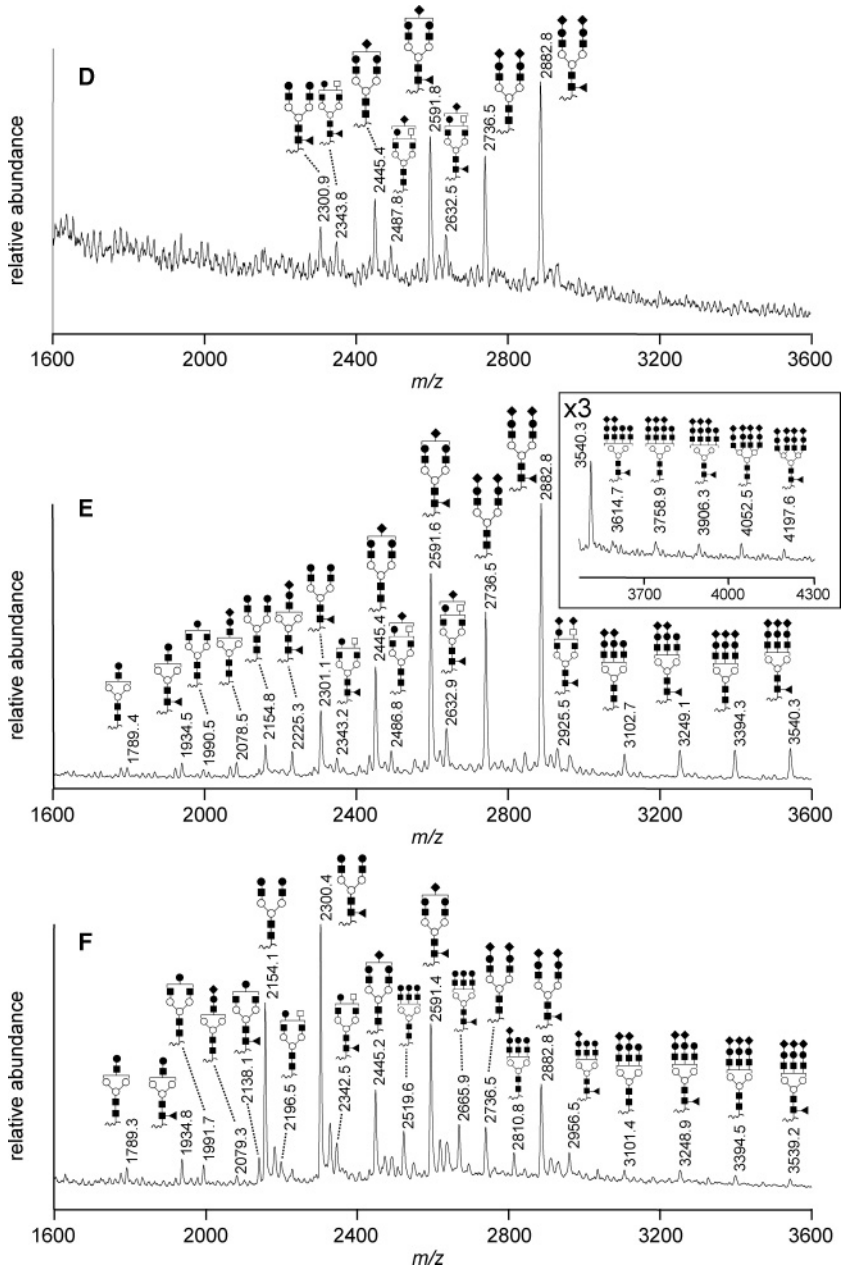


Fig. 2. (Continued.)

S-aminoethylation was performed as follows. The gel pieces dried in acetonitril were rehydrated in 100 μL of reduction buffer [10 mM dithiothreitol, 100 mM NH_4HCO_3], and left standing at 56°C for 1 h. After removal of the supernatant, the gel pieces were incubated with 30 μL of ethyleneimine (Tokyo Kasei Kogyo, Tokyo, Japan) for 30 min at room temperature in a fume hood. The gel pieces were then washed in NH_4HCO_3 and subjected to in-gel digestion with LEP.

Treatment with neuraminidases

The neuraminidases used were from *S. pneumoniae* (Sigma) and from *A. ureafaciens* (Nacalai Tesque, Kyoto, Japan). Neuraminidase digestion was applied to the electrophoretically separated proteins in the polyacrylamide gels prior to in-gel LEP digestion as follows. After washing and dehydration, the dried gel pieces were rehydrated in 100 μL of neuraminidase solution [100 mU/mL of α2,3-neuraminidase from *S. pneumoniae* in 50 mM sodium phosphate, pH 6.0, or 10 mU/mL of α2,6-neuraminidase from *A. ureafaciens* in 50 mM sodium acetate, pH 5.0] and incubated at 37°C for 2 h. The gel pieces were washed in 100 μL of 100 mM NH_4HCO_3, and dehydrated in 300 μL of acetonitrile, and subjected to in-gel LEP digestion as described above.

Purification of glycopeptides

The peptides from in-gel LEP digestion of S-aminoethylated PSA from the seminal plasma or cancer patient serum were separated by a 1.0 mm × 150 mm C30 reversed phase column

(Develosil C30-UG5, Nomura Kagaku, Seto, Japan). Following sample loading, the column was eluted with 100% solvent A for 10 min, at which time point solvent B was increased to 30% over 30 min. The eluents were collected in fractions of 50 μL each.

Mass spectrometry

Each fraction from reversed phase chromatography was reduced in volume to a few μL using a vacuum centrifuge concentrator. Equal amounts of the resulting peptide solution and a MALDI sample matrix solution [10 mg/mL of recrystallized 2,5-dihydroxbenzoic acid (Wako Pure Chemical, Osaka, Japan) dissolved in 30% acetonitrile, 0.1% trifluoroacetic acid] were mixed on the sample target. The mass spectra for obtaining glycan profiles were acquired using a MALDI time-of-flight (TOF) mass spectrometer (Voyager DE Pro, Applied Biosystems, Foster City, CA) in the linear mode. Tandem MS was carried out on a MALDI quadrupole ion trap TOF mass spectrometer (AXIMA QIT, Shimadzu, Kyoto, Japan), using argon as a collision gas for collision-induced dissociation (CID). Measurements were carried out in a positive ion mode to detect protonated molecules, $[M+H]^+$. Relative molecular mass was used in the present study.

All the experiments from sample preparation to MS shown in Scheme 1A were repeated at least twice.

Supplementary Data

Supplementary data for this article is available online at http://glycob.oxfordjournals.org

Funding

Japan Society for the Promotion of Science (B-19390093)

Acknowledgement

This work was supported by the 21st Century Center of Excellence (COE) Program.

Conflict of interest statement

None declared.

Abbreviations

ACT, α_1-antichymotrypsin; ACT–PSA, α_1-antichymotrypsin-prostate specific antigen; MS, mass spectrometry; PSA, prostate specific antigen; QIT, quadrupole ion-trap; SDS–PAGE, sodiumdodecyl sulfate–polyacrylamide gel electrophoresis; TOF, time-of-flight.

References

Bélanger A, van Halbeek H, Graves HC, Grandbois K, Stamey TA, Huang L, Poppe I, Labrie F. 1995. Molecular mass and carbohydrate structure of prostate specific antigen: Studies for establishment of an international PSA standard. Prostate. 27:187–197.

Catalona WJ, Smith DS, Ornstein DK. 1997. Prostate cancer detection in men with serum PSA concentrations of 2.6 to 4.0 ng/mL and benign prostate examination. Enhancement of specificity with free PSA measurements. JAMA. 277:1452–1455.

Catalona WJ, Partin AW, Slawin KM, Brawer MK, Flanigan RC, Patel A, Richie JP, deKernion JB, Walsh PC, Scardino PT et al. 1998. Use of the percentage of free prostate-specific antigen to enhance differentiation of prostate cancer from benign prostatic disease: A prospective multicenter clinical trial. JAMA. 279:1542–1547.

Christensson A, Laurell CB, Lilja H. 1990. Enzymatic activity of prostate-specific antigen and its reactions with extracellular serine proteinase inhibitors. Eur J Biochem. 194:755–763.

Fukuda M. 1996. Possible roles of tumor-associated carbohydrate antigens. Cancer Res. 56:2237–2244.

Jain S, Bhojwani AG, Mellon JK. 2002. Improving the utility of prostate specific antigen (PSA) in the diagnosis of prostate cancer: the use of PSA derivatives and novel markers. Postgrad Med J. 78:646–650.

Kim YJ, Varki A. 1997. Perspectives on the significance of altered glycosylation of glycoproteins in cancer. Glycoconj J. 14:569–576.

Laine A, Hachulla E, Strecker G, Michalski JC, Wieruszeski JM. 1991. Structure determination of the glycans of human-serum α1-antichymotrypsin using 1H-NMR spectroscopy and deglycosylation by N-glycanase. Eur J Biochem. 197:209–215.

Lilja H, Christensson A, Dahlén U, Matikainen MT, Nilsson O, Pettersson K, Lövgren T. 1991. Prostate-specific antigen in serum occurs predominantly in complex with α1-antichymotrypsin. Clin Chem. 37:1618–1625.

Ohyama C, Hosono M, Nitta K, Oh-eda M, Yoshikawa K, Habuchi T, Arai Y, Fukuda M. 2004. Carbohydrate structure and differential binding of prostate specific antigen to Maackia amurensis lectin between prostate cancer and benign prostate hypertrophy. Glycobiology. 14:671–679.

Okada T, Sato Y, Kobayashi N, Sumida K, Satomura S, Matsuura S, Takasaki M, Endo T. 2001. Structural characteristics of the *N*-glycans of two isoforms of prostate-specific antigens purified from human seminal fluid. Biochim Biophys Acta. 1525:149–160.

Peracaula R, Tabarés G, Royle L, Harvey DJ, Dwek RA, Rudd PM, de Llorens R. 2003. Altered glycosylation pattern allows the distinction between prostate-specific antigen (PSA) from normal and tumor origins. Glycobiology. 13:457–470.

Peter J, Unverzagt C, Hoesel W. 2000. Analysis of free prostate-specific antigen (PSA) after chemical release from the complex with α1-antichymotrypsin (PSA-ACT). Clin Chem. 46:474–482.

Prakash S, Robbins PW. 2000. Glycotyping of prostate specific antigen. Glycobiology. 10:173–176.

Shevchenko A, Tomas H, Havlis J, Olsen JV, Mann M. 2006. In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nat Protoc. 1:2856–2860.

Stamey TA, Yang N, Hay AR, McNeal JE, Freiha FS, Redwine E. 1987. Prostate-specific antigen as a serum marker for adenocarcinoma of the prostate. N Engl J Med. 317:909–916.

Stenman UH, Leinonen J, Alfthan H, Rannikko S, Tuhkanen K, Alfthan O. 1991. A complex between prostate-specific antigen and α1-antichymotrypsin is the major form of prostate-specific antigen in serum of patients with prostatic cancer: Assay of the complex improves clinical sensitivity for cancer. Cancer Res. 51:222–226.

Stratikos E, Gettins PG. 1997. Major proteinase movement upon stable serpin-proteinase complex formation. Proc Natl Acad Sci USA. 94:453–458.

Sumi S, Arai K, Kitahara S, Yoshida K. 1999. Serial lectin affinity chromatography demonstrates altered asparagine-linked sugar-chain structures of prostate-specific antigen in human prostate carcinoma. J Chromatogr B Biomed Sci Appl. 727:9–14.

Tabarés G, Radcliffe CM, Barrabés S, Ramírez M, Aleixandre RN, Hoesel W, Dwek RA, Rudd PM, Peracaula R, de Llorens R. 2006. Different glycan structures in prostate-specific antigen from prostate cancer sera in relation to seminal plasma PSA. Glycobiology. 16:132–145.

Tabarés G, Jung K, Reiche J, Stephan C, Lein M, Peracaula R, de Llorens R, Hoesel W. 2007. Free PSA forms in prostatic tissue and sera of prostate cancer patients: Analysis by 2-DE and western blotting of immunopurified samples. Clin Biochem. 40:343–350.

Tajiri M, Yoshida S, Wada Y. 2005. Differential analysis of site-specific glycans on plasma and cellular fibronectins: Application of a hydrophilic affinity method for glycopeptide enrichment. Glycobiology. 15:1332–1340.

van Halbeek H, Gerwig GJ, Vliegenthart JF, Tsuda R, Hara M, Akiyama K, Schmid K. 1985. Occurrence of the Y determinant on the *N*-glycosidic carbohydrate units of human gamma-seminoprotein. Biochem Biophys Res Commun. 131:507–514.

Villanueva J, Shaffer DR, Philip J, Chaparro CA, Erdjument-Bromage H, Olshen AB, Fleisher M, Lilja H, Brogi E, Boyd J et al. 2006. Differential exoprotease activities confer tumor-specific serum peptidome patterns. J Clin Invest. 116:271–284.

Wada Y, Tajiri M, Yoshida S. 2004. Hydrophilic affinity isolation and MALDI multiple-stage tandem mass spectrometry of glycopeptides for glycoproteomics. Anal Chem. 76:6560–6565.

Wada Y, Azadi P, Costello CE, Dell A, Dwek RA, Geyer H, Geyer R, Kakehi K, Karlsson NG, Kato K et al. 2007. Comparison of the methods for profiling glycoprotein glycans—HUPO Human Disease Glycomics/ Proteome Initiative multi-institutional study. Glycobiology. 17:411–422.