MOLECULAR FORMS OF HUMAN PROSTATE-SPECIFIC ANTIGEN IN URINE OF SUBJECTS WITH BENIGN PROSTATIC HYPERPLASIA

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Abstract - In the present study, we examined mollecular forms of urinary prostate-specific antigen (PSA), focusing on its structural complexity in general and specifically on its microheterogeneity in relation to benign prostatic hyperplasia (BPH). Gel filtration, ion-exchange chromatography, and lectin-affinity chromatography were used to characterize PSA. In comparing the binding pattern of PSA isoforms, moderate changes were observed in the relative abundance of distinct molecular subpopulations separated on lectin-affinity columns. They may be related to alteration in the position and type of linkage of fucose or sialic acid, as well as to modification of the trimannosyl core by branching of the PSA oligosaccharide chain.

Key words: Prostate-specific antigen, urine, isoforms, glycosylation, benign hyperplasia

INTRODUCTION

Prostate-specific antigen, PSA, is serine protease, which exist as an intact enzymatically active form as well as an enzymatically inactive form comprising pro-PSA, mature inact PSA, and several types of clipped PSA forms (S t e p h a n *et al.,* 2002). Besides these forms, commonly referred to as free PSA, the enzymatically active form can be found in complex with different protease inhibitors, contributing to its extreme molecular heterogeneity (S t e p h a n *et al.* 2000, 2002). Differences in its polypeptide backbone affecting the possible glycosylation site and sites important for proper formation of secondary structure lead to detection of multiple PSA isoforms having a broad range of 6 – 28 kDa of molecular mass (I s o n o *et al.,* 2002). An additional source of observed PSA heterogeneity is its glycoprotein nature. The predominant PSA form is characterized as glycoprotein having a biantennary N-linked oligosaccharide of the N-acetyllactosamine type with a sialic acid group at the end of the two branches, but differences such as the presence of a monoantennary chain are also found (B e l a n g e r *et al.,* 1995; O k a d a *et al.,* 2001). The search for specific changes in PSA glycosylation as a promising tool for differential diagnostic of benign prostatic hyperlasia (BPH) and prostate cancer, both being associated with increase in PSA concentrations, is still in progress (P r a -

k a s h and R o b b i n s, 2000; P e r c a u l a *et al.,* 2003; B a s u *et al.,* 2003; J a n k o v i ć and K o s a n o v i ć, 2005).

Proceeding from the lack of structural information about urinary PSA in the available literature, in the present study we examined glycosylation of BPH PSA, *i.e.,* prostate-specific antigen isoforms originating from the urine of subjects with benign prostatic hyperplasia. This analysis was aimed at gaining more insight into the complexity of PSA in general and specifically into microheterogeneity as related to this particular pathophysiological condition. To reach specific conclusions, our approach was based on combination of gel filtration, ion-exchange chromatography and lectin affinity chromatography employing a panel of plant lectins with different carbohydrate-binding specificities. The obtained results indicate microheterogenity of the two main PSA isoforms, as well as differences between them with respect to particular glycoforms.

MATERIALS AND METHODS

Materials

Radioiodine (^{125}I) was from the Institute of Isotopes

Co., Ltd. (Budapest, Hungary). Mouse monoclonal anti-PSA antibodies were from Medix Biochemica (Finland) and Acris Antibodies (Germany). Bovine serum albumin (BSA), mannose (Man), and fucose and N-acetylglucosamine (GlcNAc) were from Sigma (St. Louis, USA). Lactose and galactose (Gal) were from ICN Biochemicals (Cleveland, Ohio, USA). The molecular mass markers Sephadex G-100 and DEAE Sephadex A-50 were purchased from Pharmacia Fine Chemicals AB (Uppsala, Sweden). Affinity columns with immobilized plant lectins were from Vector Laboratories (Burlingame, USA). All other chemicals were reagent grade.

Sample collection and preparation

Urine was collected from subjects with BPH as diagnosed at the Institute for Urology and Nephrology, Clinical Center of Serbia, Belgrade, Serbia and Montenegro, before initiation of any treatment. Samples were clarified by centrifugation (5 min; 14000 rpm), aliquouts were made and stored at - 20°C, until processed. Prostate-specific antigen concentration in urine was determined using a solid phase immunoradiometric assay, IRMA PSA (INEP, SCG), and selected samples (PSA > 60 ug/L) were pooled and used for further characterization.

Gel filtration

Urine (0.3 ml) was passed through a Sephadex G-100 column (bed volume 40 ml) equilibrated and eluted with 0.1 M phosphate buffered saline, pH 7.2. Fractions (0.5 ml) were collected and elution monitored by measuring PSA-immunoreactivity. The column was calibrated with molecular mass standards: bovine serum albumin, 66 kDa; ovalbumin, 43 kDa; chymotrypsinogen, 25 kDa and ribonuclease, 13.7 kDa.

Ion-exchange chromatography

Urine was loaded on a DEAE Sephadex A-50 column (20 ml bed volume) equilibrated with 0.05 M TRIS-HCl buffer, pH 7.6. The column was washed free of protein and then eluted by a discontinuous gradient of NaCl: 0.05 M NaCl, 0.1 M NaCl, 0.2 M NaCl, and 1 M NaCl. Fractions (2 ml) were collected and optical density at 280 nm and PSA-immunoreactivity were recorded. Optical density was measured using a double-beam spectrophotometer of the CE 594 type (CECIL, England). The immunoreactive fractions corresponding to particular PSA isoforms were pooled, concentrated by ultrafiltration, and

dialyzed prior to labelling with ^{125}I, using the chloramine T method as described by G r e e n w o o d *et al.,* (1963).

Lectin-affinity chromatography

Lectin affinity chromatography was performed on the columns with the following immobilized plant lectins: RCA I (*Ricinus communis* agglutinin I), Con A (lectin from *Canavalia ensiformes*), WGA (wheat germ agglutinin), PHA-E (*Phaseolus vulgaris* erythroagglutinin), PHA-L (*Phaseolus vulgaris* leukoagglutinin), UEA I (*Ulex europaeus* agglutinin I), AAA (*Aleuria aurantia* agglutinin), and SNA (*Sambucus nigra* agglutinin). A common chromatographic scheme was applied to all columns, in accordance with manufacturers' instructions. The ^{125}I-PSA isoforms (0.5 ml; 300000 - 400000 cpm) were applied to each of the columns and after 3 hours of incubation at room temperature, fractions were collected and the radioactivity of each was recorded. The unbound and retarded fractions were eluted with a binding buffer (0.1 M PBS, pH 7.2), except for the Con-A column, which was washed with 0.1 M acetate buffer (pH 6.0), supplemented with 100 mM $CaCl_2$, $MgCl_2$, and $MnCl_2$. The bound fraction was specifically eluted by the addition of competitive sugars: 0.1 M mannose (for Con-A), 0.1 M lactose (for SNA), 0.1 M galactose (for RCA), 0.1 M N-acetylglucosamine (for PHA-E, PHA-L, and WGA), and 0.1 M fucose (for UEA and AAA). Finally, the tightly bound fraction was eluted by the addition of low-pH buffers: 0.1 M glycine-HCl, pH 3.0 (for RCA, PHA-E, PHA-L, RCA, AAA, and UEA) or 0.2 M acetic acid (for SNA and WGA). The Con-A column was finally eluted with 0.1 M borate bufffer, pH 8.4.

RESULTS AND DISCUSSION

The presence of PSA in urine was initially checked by immunoradiometric assay for total PSA, the results of which indicated its distribution in the broad concentration range of 0.5 – 131 µg/l, possibly due to influence of osmolality change. In order to find out the molecular forms with which this immunoreactivity is associated, the urine was subjected to gel filtration on a Sephadex G-100 column. The elution profile revealed a minor 90 kDa immunoreactive peak corresponding to molecular mass of the PSA-ACT complex, and a major 30 kDa peak corresponding to the molecular mass of free PSA (Fig. 1). Thus, urinary PSA was confirmed to be predominantely in its free form as indirectly suggested in previous reports (P a n n e k *et al.,* 1997).

cpm
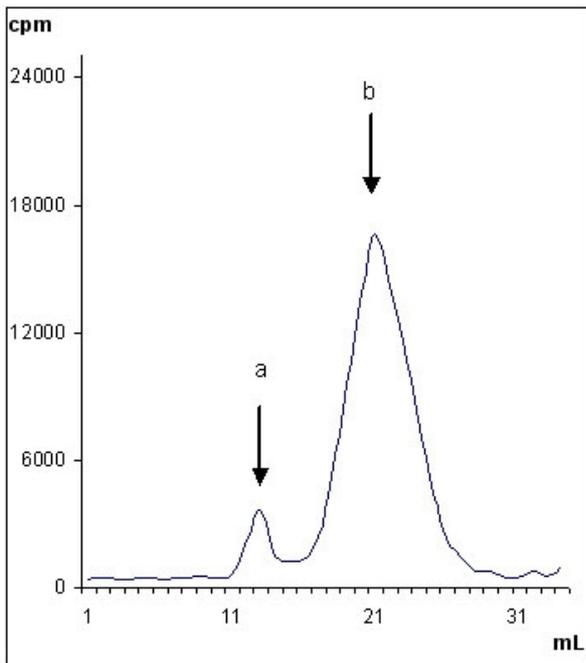
Fig. 1. Urinary BPH PSA immunogram from Sephadex G-100 column. Arrows indicate position of: a) 90 kD peak (PSA-ACT complex) and b) 30 kD PSA (free form).

The molecular heterogeneity of urinary PSA was further examined by anion-exchange chromatography. Under the emloyed experimental conditions, urinary BPH PSA were retained on DEAE Sephadex A-50 and subsequentlly separated into four isoforms by the elution with increasing concentrations of NaCl (data not shown). The main PSA-immunoreactive isoforms were eluted by 0.05 M NaCl and 0.1 M NaCl (in a ratio of 1:0.6). In addition, two minor PSA-immunoreactive forms (<25% of total) eluted by 0.2 M NaCl and 1 M NaCl were also detected. Each of them exhibited broad elution profiles indicating that PSA isoforms may be composed of distinct molecular subpopulations. The differences observed in the number and relative abundance of particular urinary isoforms in benign hyperplasia as compared to the normal condition are presumed to be related not only to the examined source, but also to a specific pathophysiological process, since they correspond to previously observed changes in PSA-isoelectric patterns. Thus, PSA from normal human seminal plasma can be separated into five isoforms with pH in the range of 6.7 – 7.2, whereas a shift in isoelectric point values was observed in PSA from sera or tissue of subjects with cancer or BPH (Z h a n g *et al.,* 1995; H u b e r *et al.,* 1995; H i l z *et al.,* 1999).

The biological importance and structural character-

istics responsible for the existence of different PSA isoforms are not fully understood. It has been reported that neuraminidase treatment of distinct PSA isoforms causes a shift in their pH towards more basic values and reduces the differences between them (Z h a n g *et al.,* 1995). Thus, apart from differences in the protein backbone related to internal peptide bond cleavage, carbohydrate composition as a possible cause must not be overlooked.

In the present study, glycosylation as a factor contributing to total heterogeneity of urinary PSA isoforms was investigated by lectin affinity chromatography. Lectins are widely used as a tool for isolation and characterization of different glycoconjugates (K o b a t a and E n - d o , 1992; C u m m i n g s, 1994). By choosing this method and working with purified isoforms, we could gain insight into the microheterogeneity of each of the isoforms, *i.e.,* separate PSA glycoforms bound with low affinity (such as those eluted with a binding buffer), with intermediate affinity (such as those eluted with a competitive sugar at 0.1 – 0.3 M concentration), and with high affinity (such as those eluted with a low-pH buffer). On the other hand, the carbohydrate-binding specificity of the eight plant lectins tested enabled us to infer the type of linkage and composition of the PSA oligosaccharide chain with respect to the terminal glycosylation as well as inner and outer chain moieties, which characteristics are important for comparison with known structural data (B e l a n g e r *et al.,* 1995).

The two main urinary PSA isoforms (separated by 0.05 M NaCl and 0.1 M NaCl) were characterized after being labelled with radioiodine. The representative profiles of elution from the columns with immobilized plant lectins are shown in Fig. 2. In all cases, most of the applied radioactivity was recovered by elution with buffer lacking sugar. According to the differential elution positions on the same column, the corresponding eluents were divided into a non-interacting fraction and a fraction that was retarded in elution (referred to zero and total column volume). Monotonic or retarded elution fronts (as a trailing edge or as separated) were observed depending on the lectin used, but in accordance with their known binding pattern (K o b a t a and E n d o, 1992; C u m - m i n g s , 1994). Thus, the least microheterogeneity was observed on the Con A column, the gratest on the PHA-E column. The PSA fractions bound with either intermediate or high affinity were observed on all columns, but compared to the unbound and retarded fractions, their ratios were low.
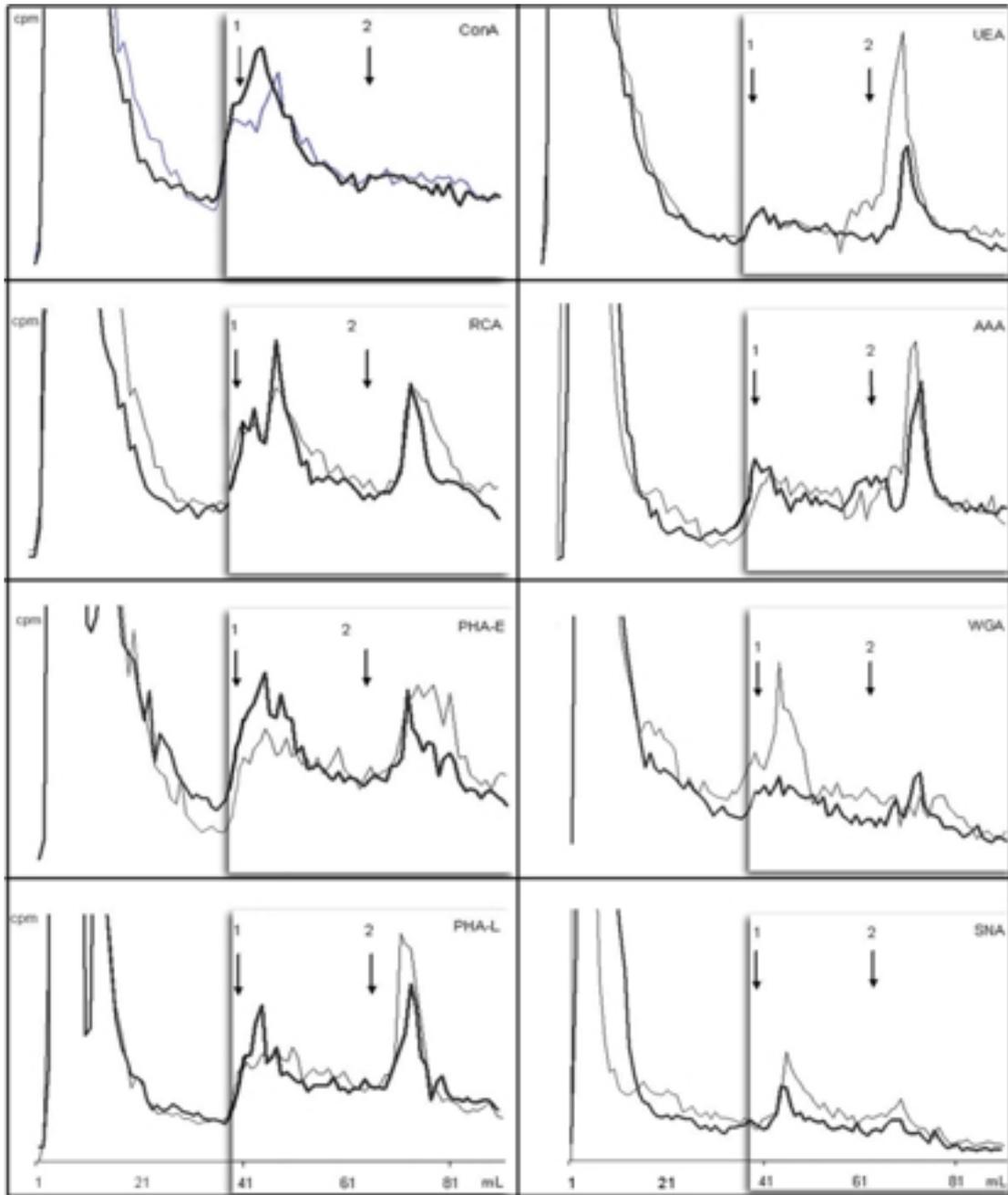
Fig. 2. Lectin-affinity chromatography of [125] I-PSA isoforms separated by ion-exchange chromatography on DEAE A-50, using: 0.05 M NaCl (—) and 0.1 M NaCl (—). The elution was monitored by measuring radioactivity in each fraction. Arrows indicate the start of elution with 1) sugar and 2) low pH buffer, as described in Material and Methods. For better insight into the details of elution profiles, parts of the graphs (shown in the outline boxes) are magnified.

Although PSA is a glycoprotein having low carbohydrate content (8-12%), it can also exist as a non-glycosylated form (B e l a n g e r *et al.,* 1995; T a n a k a *et al.,* 2000). Available data on lectin affinity chromatography of PSAs from different preparations indicated that plant lectins (except for ConA) exhibit low PSA binding (S u m i *et al.,* 2001). Compared to previous results on seminal plasma PSA and available literature data, a decrease in lectin binding to urinary BPH PSA was evident, which is in agreement with the general expectation of decrease in disease-accociated PSA forms (B a r a k *et al.,* 1989; S a m a d i *et al.,* 1999; S u m i *et al.,* 2001; K o - s a n o v i ć and J a n k o v i ć, 2005). In comparing lectin-binding patterns of the two main urinary PSA isoforms, moderate changes in the relative abundance of distinct molecular subpopulations separated on UEA, PHA-E, and PHA-L columns can be seen, without any other striking differences. By referring to the carbohydrate-binding specificities of lectins used in this investigation, the characteristic properties of the oligosaccharide chain of urinary BPH PSA can be assessed. They are presumed to be related to the position and type of linkage of fucose or sialic acid, as well as to modification of the trimannosyl core by branching. Thus, Con A (which has high affinity for the high mannose type or hybrid type of oligosaccharide chains with fucose linkages to the innermost GlcNAc) showed low affinity binding to the examined BPH PSA isoforms, suggesting modification of the trimannosyl core [Man α1-6 (Man α1,3) Man] by the presence of bisecting GlcNAc or higher branching and formation of multiantennary chains (G o l d s t e i n and P o r e t z, 1986; K o b a t a and E n d o, 1992; C u m - m i n g s, 1994). Strong retardation of urinary BPH PSA on PHA-E (specific for the complex type of oligosaccharide chains containing bisecting GlcNAc β1,4 linkage) and on PHA-L (specific for the multiantennary complex type of oligosaccharide chains with GlcNAc β1,6 branches) may support these findings (G o l d s t e i n and P o r e t z, 1986; K o b a t a and E n d o, 1992; C u m - m i n g s, 1994). Low-affinity binding of urinary BPH PSA isoforms to fucose-specific lectins (AAA and UEA) and sialic acid-specific lectins (SNA and WGA) was also found. It is reported that 80% of normal PSA glycan is core fucosylated, but α1,2-linked outer fucose can be detected (P e r a c a u l a *et al.,* 2003). This can also hold true in the case of urinary BPH PSA, to judge from the AAA and UEA-binding patterns. Thus, AAA binds with high affinity to core fucosylated N-linked structures, whereas many structures containing outer chain fucose substitutes do not bind, but are retarded on a AAA-col-

umn (Y a m a s h i t a *et al.,* 1985; C u m m i n g s, 1994). They can be bound to UEA, being specific for α1,2-linked outer fucose residues (Y a m a s h i t a *et al.,* 1985; C u m m i n g s, 1994). As for sialylation, normal PSA glycans are found to be di- or monosialylated and both types of linkages (α2,6 and α2,3) are presumed to be present (P e r a c a u l a *et al.,* 2003). Based on the binding pattern of SNA (which is specific for sialic acid α2,6 Gal), WGA (which has a low-affinity binding site for sialic acid), and RCA I (which specifically recognizes βGal residues, with preference for terminal sugar in β1,4 linkage), urinary BPH PSA is presumed to be not fully silaylated or to have a high ratio of sialic acid α2,3 linkage (G o l d s t e i n and P o r e t z, 1986; C u m m i n g s, 1994).

It is well known that different physiological and pathological conditions are associated with alteration of glycosylation affecting the biochemical and functional characteristics of particular glycoproteins (B r o c k - h a u s e n and K u h n s, 1997). The results obtained in the present study indicate high microheterogeneity of urinary BPH PSA isoforms and a difference in the glycoform pattern compared to the normal physiological condition. Changes in sugar chain characteristics as a potential marker of PSA origin therefore deserve attention, and further work needs to be done on formulation of assays for their detection.

REFERENCES

Barak, M., Mecz, Y., Lurie, A., and *Gruener, N.* (1989). Binding of serum prostate antigen to concanavalin A in patients with cancer or hyperplasia of the prostate. *Oncology*, **46,** 375-357.

Basu, P.S., Majhi, R., and *Batabyal, S.K.* (2003). Lectin and serum-PSA interaction as a screening test for prostate cancer. *Clin. Biochem.* **36,** 373-376.

Belanger, A., Halbeek, H., Graves, H.C., Grandbois, K., Stamey, T.A., Huang, L., Poppe, I., and *Labrie, F.* (1995). Molecular mass and carbohydrate structure of prostate-specific antigen: studies for establishment of an international PSA standard. *Prostate*, **27,** 187-97.

Brockhausen, I., and *Kuhns, W.* (1997). *Glycoproteins and Human Disease,* Springer –Verlag, Heidelberg, 1997.

Cummings, R.D. (1994). Use of lectins in analysis of glycoconjugates. *Methods Enzymol.* **230,** 66-86.

Goldstein, I.J., and *Poretz. R.D.* (1986). Isolation, physicochemical

characterization and carbohydrate-binding specificities of lectins. In: *Lectins: Properties, Functions and Applications in Biology and Medicine* (Eds. I. E. Liener, N. Sharon, I. J. Goldstein), Academic Press, London.

Greenwood, P.C., Hunter, M.W., and Glover, S. (1963). The preparation of ^{131}I labelled growth hormone of high specific radioactivity. *Biochem J.* **89,** 114-120.

Hilz, H., Noldus, J., Hammerer, P., Buck, F., Luck, M., and Hulan, H. (1999). Binding of serum prostate antigen to concanavalin A in patients with cancer or hyperplasia of the prostate. *Eur Urol.* **36,** 286-292.

Huber, P.R., Schimid, H.P., Mattarelli, G., Strittmatter, B., Steenburg, G.J., and Maurer, A. (1995). Serum free prostate specific antigen: isoenzymes in benign hyperplasia and cancer of the prostate. *Prostate,* **27,** 212-219.

Isono, T., Tanaka, T., Kageyama, S., and Yoshiki, T. (2002). Structural diversity of cancer-related and non-cancer related prostate-specific antigen. *Clin Chem.* **48,** 2187-2194.

Jankovic, M., and Kosanovic, M. (2005). Glycosylation of urinary prostate-specific antigen in benign hyperplasia and cancer: assessment by lectin-binding patterns. *Clin. Biochem.* 38, 58-65.

Kobata, A., and Endo, T. (1992). Immobilized lectin columns: useful tools for the fractionation and structural analysis of oligosaccharides. *J Chromatogr.* **597,** 111-122.

Kosanovic, M., and Jankovic, M. (2005). Sialylation and fucosylation of cancer-associated prostate-specific antigen. *J BUON.* **10,** 247-250.

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

Pannek, J., Rittenhouse, H.G., Evans, C.L., Finlay, J.A., Bruzek, D.J., Cox, J.L., Chan, D.W., Subong, E.N., and Partin, A.W. (1997). Molecular forms of prostate specific antigen and human kallik-

rein 2 (hK2) in urine are not clinically useful for early detection and staging of prostate cancer. *Urology,* **50,** 715-721.

Peracaula, R., Tabares, G., Royle, L., Harvey, D.J., Dwek, R.A., Rudd, P.M. and Lorens, R. (2003). Altered glycosylation pattren allows the distinction between prostate-specific antigen (PSA) from normal and tumor origins. *Glycobiology,* **13,** 457-470.

Prakash, S., and Robbins, P.W. (2000). Glycotyping of prostate specific antigen. *Glycobiology,* **10,** 173-176.

Samadi, A.A., Davidson, S.D., Mordente, J.A., Choudhury, M.S., Tazaki, H., Mallouh, C., and Komo, S. (1999). Differential glycosylation of cellular prostate specific antigen and the regulatory mechanism of its secretion. *Mol Urol.* **3,** 147-152.

Stephan, C., Jung, K., Lein, M., Sinha, P., Schnorr, D., and Loening, S.A. (2000). Molecular forms of prostate-specific antigen and human kallikrein 2 as promising tools for early diagnosis of prostate cancer. *Canc Epidem. Biomarkers& Prevention.* **9,** 1133-1147.

Stephan, C., Jung, K., Diamandis, E., Rittenhouse, N.G., Lein, M., and Loening, S.A. (2002). Prostate-specific antigen, its molecular forms, and other kallikrein markers for detection of prostate cancer. *Urology,* **59,** 2-8.

Sumi, S., Arai, K., and Yoshida, K. (2001). Separation methods applicable to prostate cancer diagnosis and monitoring therapy. *J Chromatogr B Biomed Sci Appl.* **764,** 445-455.

Tanaka, T., Isono, T., Yoshiki, T., Yuasa, T., and Okada, Y. (2000). A novel form of prostate-specific antigen transcript produced by alternative splicing. *Cancer Res.* **60,** 56-59.

Yamashita, K., Kochibe, N., Ohkura, T., Ueda, I., and Kobata, A. (1985). Fractionation of L-fucose-containing oligosaccharide on immobilized *Aleuria aurantia* lectin. *J Biol Chem.* **8,** 4688-6393.

Zhang, W.M., Leinonen, J., Kalkkinen, N., Dowell, B., and Stenman, U. (1995). Purification and characterization of different molecular forms of prostate-specific antigen in human seminal fluid. *Clin. Chem.* **41,** 1567-73.

МОЛЕКУЛСКЕ ФОРМЕ ХУМАНОГ СПЕЦИФИЧНОГ АНТИГЕНА ПРОСТАТЕ У УРИНУ ОСОБА СА БЕНИГНОМ ХИПЕРПЛАЗИЈОМ

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У овом раду су изучаване молекулске форме уринарног специфичног антигена простате (PSA) са циљем да се добије увид у његову структурну комплексност, уопште, и специфично, у микрохетерогеност повезану са бенигном хиперплазијом простате (BPH). PSA је испитиван гел филтрацијом, јоноизмењивачком хроматографијом и лектинском афинитетном хроматографијом. Поређењем профила везивања PSA изоформи, запажене су умерене разлике у релативном садржају посебних молекулских "субпопулација", раздвојених на лектинским колонама. Оне би се могле довести у везу са променама у положају и типу везе фукозе или сијалинске киселине као и са модификацијама триманозилног језгра у смислу гранања олигосахаридног ланца PSA.