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DIFFERENT MOLECULAR FORMS OF UNCOMPLEXED PROSTATE SPECIFIC ANTIGEN (PSA) SHOW SIMILAR IMMUNOREACTIVITIES

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ABSTRACT

PSA exists in multiple molecular forms in serum, with the majority complexed to proteinase inhibitors such as α_1-antichymotrypsin and α_2-macroglobulin. The uncomplexed, or "free" forms of PSA represent a very heterogenous distribution of molecular isoforms. It has been suggested that these variations in uncomplexed PSA may cause differences in their immunologic characteristics which may lead to analytical differences between various PSA assays. We report that various isoforms of uncomplexed PSA purified from seminal fluid as previously described show no differences in relative immunoreactivity and demonstrate equimolar behavior as measured by the TOSOH AIA®-600 assay, which is a PSA assay based upon monoclonal PSA and monoclonal detecting antibodies (mono-mono). Furthermore, we show that carbohydrate side-chain modification does not change the equimolar immunoreactivity of these isoforms.

KEY WORDS: prostate specific antigen, PSA, PSA isoforms

Prostate-specific antigen (PSA) is a 30 kDa glycoprotein[1] found mainly in seminal fluid which has become a unique serum tumor marker for the early detection and management of prostate cancer.[2,3] Most of the serum PSA in patients with prostate disease is complexed with proteinase inhibitors such as α_1-antichymotrypsin and α_2-macroglobulin.[4–6] However, uncomplexed ("free") forms of PSA also exist in serum and represent a very heterogenous distribution of molecular isoforms, including internally-cleaved ("nicked") PSA, pro-PSA, and various glycosylated variants.[4,7]

Although there are numerous commercial assays available to measure serum free and total PSA levels, differences in immunorecognition exist between these assays, especially to the uncomplexed forms of PSA.[8,9] These differences have mainly been thought to be due to differences in immunorecognition between monoclonal- and polyclonal-antibody assay formats, possibly related to variations in the immunologic characteristics of different molecular isoforms of uncomplexed PSA.

In this study we have investigated the issue of variable immunoreactivity between different PSA isoforms by isolating various molecular forms of uncomplexed PSA and characterizing their individual immunoreactivity as measured by several commercially-available PSA assays.

MATERIALS AND METHODS

Materials. PSA was purified from human seminal plasma as previously described.[1] Purified α_2-macroglobulin was purchased from Athens Research Technology (Athens, CA). Resource 15Q chromatography media and XK16/20 column system were obtained from Pharmacia (Uppsala, Sweden). Molecular mass calibration proteins, isoelectric focusing ready gels, and other reagents for gel electrophoresis were obtained from Bio-Rad Labs (Hercules, CA). Synthetic chromogenic substrate, MeO-Suc-Arg-Pro-Tyr-NH-nitroanilide (S-2586) and H-D-Pro-Phe-Arg-NH-nitroanilide (S-2302), were purchased from Kabi Pharmacia (Franklin, OH), Centrifugal force concentrators (Centricon-10) were obtained from Amicon (Beverly, MA). Neuraminidase was purchased from Sigma (St. Louis, MO). Immunoassay reagent kits were generous gifts from TOSOH Medics (Foster City, CA) and Diagnostic Products Corporation (Los Angeles, CA) manufacturers.

Methods: purification. PSA was initially purified from human seminal plasma in our lab as previously described.[1] The PSA was then dialyzed against three changes of one L volumes of 10 mmol/l. Tris-HCl, pH 8.0, containing 2 mmol/l. benzamidine (buffer A). The dialyzed PSA was clarified by centrifugation (60,000 g, 30 min., 4C) and applied to an XK 16/20 column containing a 10 ml. bed volume of Resource 15Q chromatography media and equilibrated with buffer A.[7] The column was mounted to Econo system (Bio-Red Labs, Hercules, CA) and washed with 10 bed volumes of buffer A at a flow rate of 0.8 ml./min. The bound proteins were then eluted with a linear gradient of 60 ml. of buffer A and 60 ml. buffer B (buffer A plus 0.15 mol/l. NaCl) at 0.8 ml./min., collecting 2-ml. fractions, essentially as previously described.[7] The PSA isoforms were concentrated with centrifugal force concentrators (Centricon-10), and washed 40-fold with 10 mmol/l. Tris-HCl buffer, pH 7.3 containing 150 mmol/l. NaCl before further characterization.

Electrophoretic methods. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli[10] with modification.[11] SDS-PAGE was also performed under reducing conditions by the addition of 1% w/v β-mercaptoethanol to samples. To visualize the protein bands, we stained the gels with Coomassie Brilliant Blue R-250. Isoelectric focusing (IEF) was performed with IEF ready gels in the pH range of 3-10 according to the instructions of the manufacture. Proteins were stained with crocein scarlet and Coomassie Blue R-250.

Enzyme assay. The enzymatic activity of PSA was measured by adding 30 μg. of PSA to 0.5 ml. of assay buffer (0.2 mol/l. Tris-HCl buffer, pH 7.8) containing synthetic chromogenic substrates. Substrate S-2586 (final concentration 0.14 mmol/l.) was used to measure chymotrypsin-like activity and substrate S-2302 (final concentration 0.25 mmol/l.) was used to measure trypsin-like activity. The reaction was terminated by adding 25 μl. of glacial acetic acid. The products of hydrolysis were measured spectrophotometrically at 405 nm.[12] The PSA concentration was determined by an absorbtivity of 1.84 ml. $\times$ mg.$^{-1}$ $\times$ cm.$^{-1}$.[13]

PSA complexation. PSA was incubated at a 1:2 ratio with

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either purified α_2-macroglobulin or α_1-antichymotrypsin in complexation buffer (0.05 mol/l. Tris-HCl buffer, pH 7.4 containing 0.05 mol/l. NaCl) at 37C for 2 hours. Complexation was terminated by the addition of stop buffer (final concentration 0.2 mol/l. sodium acetate, pH 5.6 containing 10 mg./ml. BSA). The percentage of PSA bound to α_2-macroglobulin was derived from PSA of control group minus PSA of reaction group and then divided by control group; the percentage of PSA bound to α_1-antichymotrypsin was derived by measuring total and free PSA by DPC assays.

Immunoassays. Purified PSA isoforms were diluted with PBS buffer containing 1% BSA to the proper range of the PSA assay. The immunoreactivities of PSA were measured in the TOSOH AIA®-600, DPC Immulite® Third Generation and DPC Immulite® Free PSA assays according to the instructions of the manufacturers.

Carbohydrate side-chain modifications of PSA isoforms. To cleave oligosaccharide chains, purified PSA isoforms were reacted with neuraminidase at a 1:5 (w/w) ratio in 100 mM Tris-HCl, pH 7.3 at 37C for four hours. Control samples consisted of purified PSA isoforms incubated without neuraminidase in 100 mM Tris-HCl, pH 7.3 at 37C for four hours. After incubation, equivalent amounts of each isoform (treated with and without neuroamidase) were measured by TOSOH assay. The same samples were run on IEF gel electrophoresis as described above.

RESULTS

Purification of PSA isoforms. Figure 1 shows the elution profile of PSA purified by the Sensabaugh method[4] as it is fractionated by strong anion-exchange chromotography as previously described by Zhang.[7] The PSA isoforms were pooled into five distinct fractions, A-E, which showed the following distribution (as % total PSA recovered): A (32%), B (29%), C (8%), D (9%), E (23%).

Gel electrophoresis. The purified PSA and the five PSA isoforms of Resource 15Q column chromatography (peaks A-E, figure 1) show single bands at approximately 30 kDa by SDS-PAGE (figure 2,A). Under reducing conditions, pooled fraction A shows a single band at approximately 30 kDa, while all other fractions show multiple bands over a wide molecular weight range (figure 2, B). Isoelectric focusing gel electrophoresis demonstrates a single band at approximately pH 6.9 for fraction A, while all other fractions show multiple bands ranging from pH 4.8 to pH 7.5 (figure 2, C).

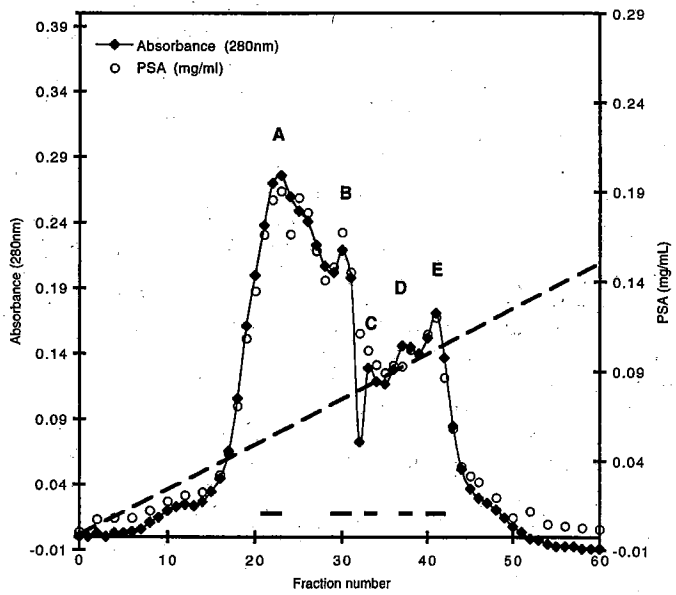

FIG. 1. Anion-exchange (Resource 15Q) chromatography of PSA. PSA concentration was determined by TOSOH AIA®-600 assay (○), and protein was determined by spectrophotometric absorbance (280nm) (◆). Linear NaCl gradient from 0–0.15 mol/l. used for protein elution is indicated by dotted line. Fractions which were combined for further characterization are indicated by bold lines and are labeled from A-E.

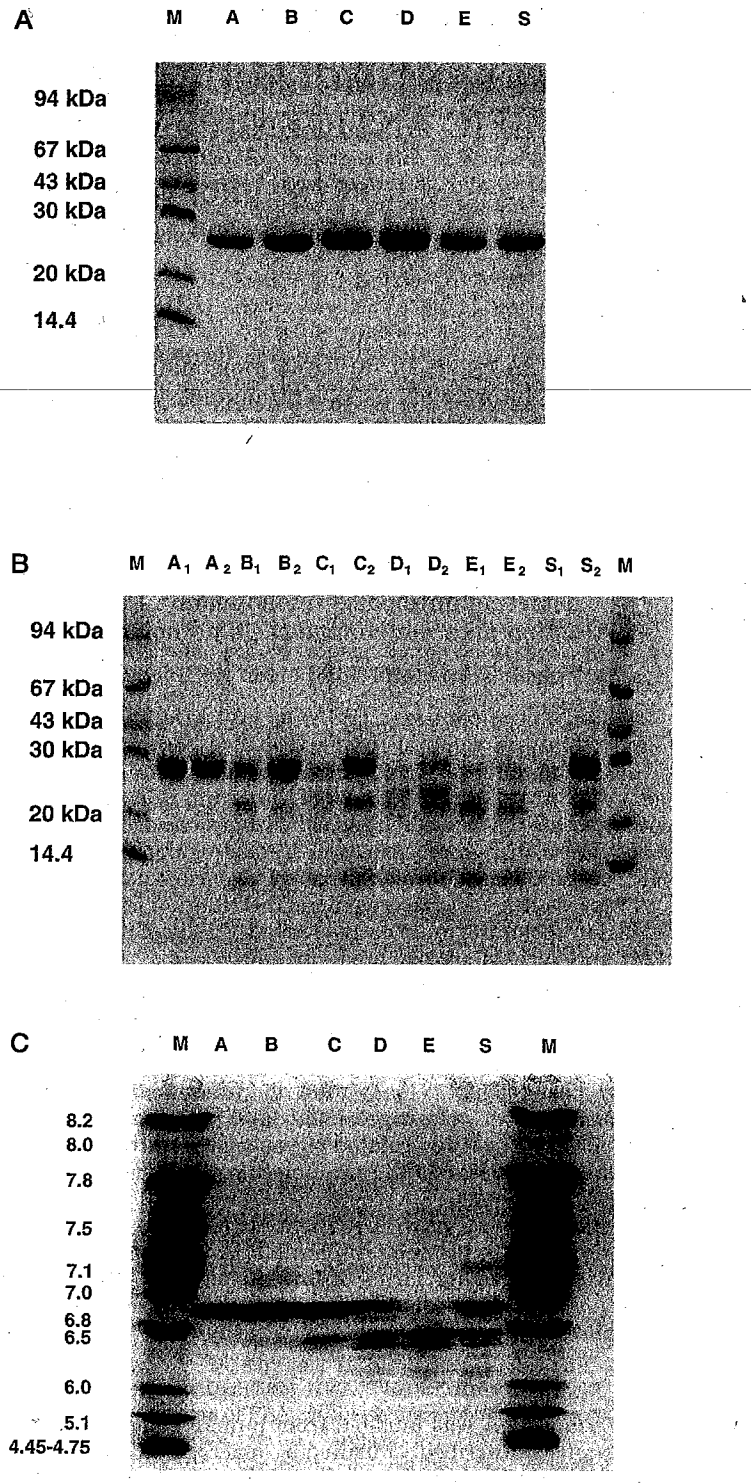

FIG. 2. SDS gel electrophoresis of PSA isoforms under reducing and non-reducing conditions. *A*, SDS-PAGE was performed at non-reducing conditions. Lanes A to E are PSA isoforms A-E from pooled peaks fractions of Figure 1; Lane S is sample prior to anion-exchange chromatography. *B*, two preparations of PSA isoforms A-E were electrophoresed at reducing conditions. Two preparations are labeled with footnote numbers 1 or 2 on top of gel. *C*, isoelectric focusing gel electrophoresis of PSA isoforms A-E in pH 3–10 range, Lane S is sample prior to anion-exchange chromatography performed by IEF gels purchased from Bio-Red.

Enzyme activity. Chymotrypsin- and trypsin-like activities of the isoforms vary considerably, with maximum activity contained within fraction A (592 $A_{405} \cdot min^{-1} \cdot g^{-1}$), and decreasing activities in fractions B (486 $A_{405} \cdot min^{-1} \cdot g^{-1}$), C (381 $A_{405} \cdot min^{-1} \cdot g^{-1}$), D (281 $A_{405} \cdot min^{-1} \cdot g^{-1}$), and E (243 $A_{405} \cdot min^{-1} \cdot g^{-1}$) (figure 3). Fractions also varied by type of activity, with Fraction A showing 98% chymotrypsin and 2% trypsin activity (98/2), and subsequent fractions showing increasing trypsin-like activity: B (92/8), C (77/23), D (62/38), and E (86/14).

Inhibitor binding. The PSA isoforms also vary significantly in the degree and type of complexation to ACT and MG (figure 4). Fractions A and B show the highest overall complexation with 78% complexed to MG and 36% to ACT (78/36) in A, and fraction B (79/33), and decreasing complexation in C (66/32), D (40/22) and E (28/16).

Immunoreactivity of PSA isoforms measured with Tosoh and DPC assay. Figure 5 shows the immunoreactivity of different PSA isoforms as measured by the mono-mono and mono-poly PSA assays. The mono-mono assay shows an equimolar response to each isoform relative to each other and an absolute equimolar response relative to the A280 calculated PSA. The mono-poly-based assays show higher immunoreactivity relative to the A_{280} PSA. Most mono-poly-based assays have adjustment for measuring serum PSA in which PSA-ACT complex is the major form; not surprisingly free PSA (PSA isoforms) were read higher by DPC than Tosoh. DPC assays react quite differently with the PSA isoforms. Probably the polyclonal antibody tracer has a different affinity to the PSA isoforms.

Carbohydrate side-chain modifications of PSA isoforms. The PSA isoforms exhibit equimolar behavior in the Tosoh assay when compared to neuraminidase-treated samples. The ratio of the measured PSA concentration using equimolar amounts (calculated from absorbtivity) of neuraminidase-treated and untreated samples is near unity for all isoforms studied. Additionally, IEF gel electrophoresis demonstrates distinct band-shifting of neuraminidase-treated samples, which is consistent with carbohydrate side-chain modification of the treated samples (gel not shown).

DISCUSSION

The procedures for purification of PSA from human prostatic tissue or seminal fluid are well documented.[1,14–18] However, purified PSA is heterogeneous. It contains mature, intact molecular forms of PSA and nicked PSA, and trace amounts of human kallikrein 2 (HK2) which share 80% homogeneity with PSA; it may also contain pre-form PSA or zymogen form of PSA.[19] Different PSA isoforms and PSA-ACT complex have been studied by several groups.[20–22] All forms of PSA and HK2 may be recognized by commercial immunoassays for PSA. Although we showed that most serum free PSA has an N-terminal sequence of IVGGW identical to mature PSA,[23] we have been unable to further characterize serum f-PSA because it could only be partially purified. To understand the nature of free PSA, we investigated the PSA isoforms of seminal fluid. Our laboratory has traditionally purified PSA from human seminal fluid using the Sensabaugh method, which yields a heterogeneous distribution of different molecular forms of uncomplexed PSA.[1] Although others have described alternative methods of purifying PSA isoforms from seminal fluid, we found it more convenient to add an adjunct strong anion-exchange column

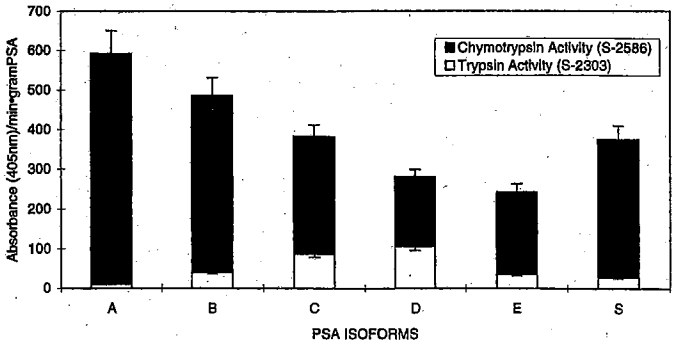

FIG. 3. Relative enzyme activities of PSA isoforms. Enzyme assays of PSA isoforms were performed using synthetic substrates S-2586 and S-2302. Reaction was carried out at 37C in tris buffer, pH 7.8, and stopped by adding glacial acetic acid.

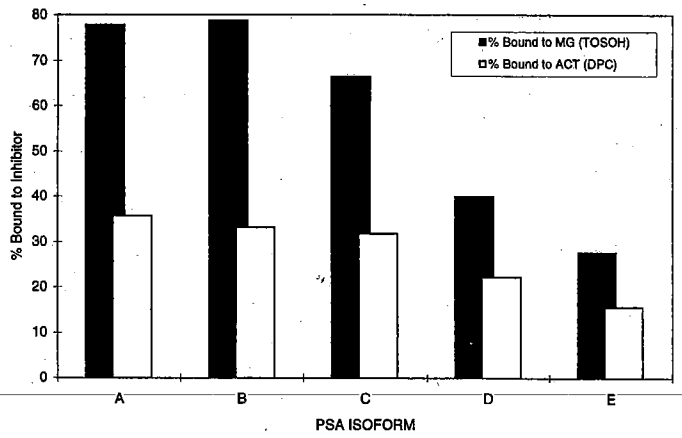

FIG. 4. PSA isoform inhibitor studies. PSA isoforms A-E from Resource 15Q column were incubated with either α_1-antichymotrypsin or α_2-macroglobulin in molar ratio of 1 to 2 at 37C for 2 hours; reactions were terminated by addition of sodium acetate buffer, pH 5.6. PSA isoforms A-E were incubated in same method without inhibitors as control groups. Reaction mixtures then were diluted to proper assay range by sodium acetate buffer, pH 5.6 containing 1% BSA and assayed by Tosoh and DPC assays. Percentage bound to α_2-macroglobulin was derived from PSA of control group minus PSA of reaction group and then divided by control group; percentage bound to α_1-antichymotrypsin was derived by measuring total and free PSA by DPC assay.

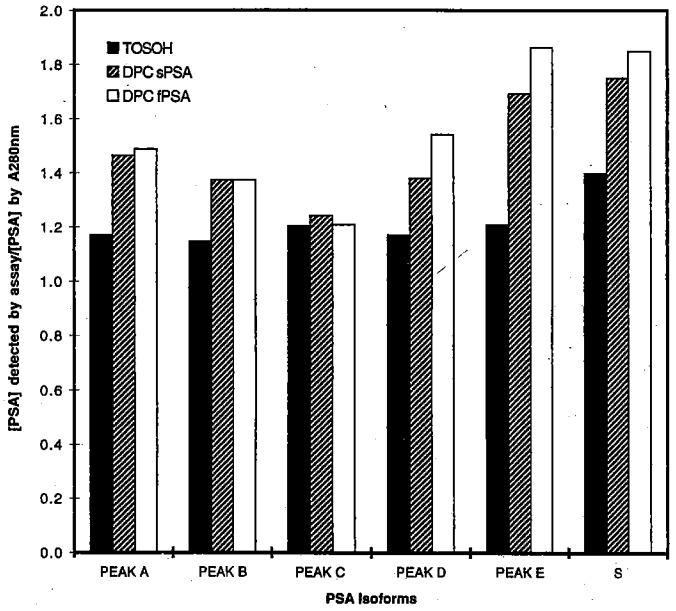

FIG. 5. Immunoreactivity of different molecular forms of PSA as measured by TOSOH AIA®-600, DPC Immulite® Third Generation and DPC Immulite® Free PSA assays. PSA isoforms (labeled peaks A-E), seminal fluid (labeled with S) were diluted with 1% BSA to working detection range of each assay. Results are presented as ratio of assayed PSA concentration to known PSA based on A280 nm. (extinction coefficient 1.84).

to the existing Sensabaugh method.[1,7] We obtained five unique molecular forms of PSA, which is consistent with a number of previous reports.[1]

We have demonstrated that these various fractions have discrete and unique biochemical profiles. SDS-PAGE analysis under reducing conditions suggests the presence of nicked forms of PSA infractions B-E, while IEF gel electrophoresis confirms the presence of major protein bands with unique pl values. Enzyme activity measured by synthetic substrates also show that highly active PSA, essentially free of trypsin-activity, can be obtained as previously observed.[7] Finally, complexion studies with protein as inhibitors ACT and MG confirm the decreased complexion affinity exhibited by nicked forms of PSA.[7]

To test the hypothesis that different molecular forms of uncomplexed PSA may have different immunologic properties, we examined the immunoreactivity of our purified PSA isoforms in several commercially available PSA assays. Interestingly, our results indicate that there is essentially no difference in immunoreactivity between PSA isoforms when measured by Tosoh AIA-600 PSA assay, a test that uses monoclonal capture antibody for PSA and monoclonal tracer antibody. These results suggest that the epitope of PSA recognized by Tosoh antibodies is probably not significantly changed by internal cleavage or other modifications (glycosylation) which can cause differences between PSA isoforms. The DPC assay demonstrated consistently higher PSA values. These results, however, are consistent with previous observations of free PSA measured by assays which are based upon monoclonal antibody as capture and polyclonal antibodies as tracer. Finally, we show that carbohydrate side-chain modification with neuraminidase causes changes in the IEF gel electrophoresis profiles of PSA isoforms, but does not affect their equimolar immunoreactivity as measured by the Tosoh assay.

These results suggest that carbohydrate side-chains may not be important factors in determining epitope specificity for the anti-PSA antibodies used in the Tosoh AIA-600 PSA assay and suggest that such assays would be less likely to exhibit fluctuations due to variations in the molecular forms of complexed or free PSA.

CONCLUSION

Our observations suggest that the various uncomplexed isoforms which comprise the heterogeneous distribution of PSA isolated from human seminal fluid using the modified. Sensabaugh method behave with equal immunoreactivity regardless of internal nicking or carbohydrate side-chain modifications. These observations have important implications for the understanding of free PSA including our observation that the free forms of PSA in seminal fluid react with equimolarity.

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REFERENCES

1. Sensabaugh, G. F. and Blake, E. T.: Seminal plasma protein p30: simplified purification and evidence for identity with prostate specific antigen. J. Urol., **144:** 1523, 1990.
2. Stamey, T. A., Yang, N., Hay, A. R., McNeal, J. E., Freiha, F. S. and Redwine, E. A.: Prostate-specific antigen as a serum marker for adenocarcinoma of the prostate. N. Engl. J. Med., **317:** 909, 1987.
3. Catalona, W. J., Smith, D. S., Ratliff, T. L., Dodds, K. M., Coplen, D. E., Yuan, J. J., Petros, J. A. and Andriole, G. L.: Measurement of prostate-specific antigen in serum as a screening test for prostate cancer. N. Engl. J. Med., **324:** 1156, 1991.
4. Christensson, A., Laurell, C. B. and Lilja, H.: Enzymatic activity of prostate-specific antigen and its reaction with extracellular serine proteinase inhibitors. Eur. J. Biochem., **194:** 755, 1990.
5. Stamey, T. A., Chen, Z. and Prestigiacomo, A. F.: Serum prostate specific antigen binding α_1-antichymotrypsin: influence of cancer volume, location and therapeutic selection of resistant clones. J. Urol., **152:** 1510, 1994.
6. Chen, Z., Komatsu, K., Prestigiacomo, A. F. and Stamey, T. A.: Addition of purified prostate specific antigen to serum from female subjects: studies on the relative inhibition by α_2-macroglobulin and α_1-antichymotrypsin. J. Urol., **156:** 1357, 1996.
7. Zhang, W. M., Leinonen, J., Kalkkinen, N., Dowell, B. and Stenman, U. H.: Purification and characterization of different molecular forms of prostate-specific antigen in human seminal fluid. Clin. Chem., **41:** 1567, 1995.
8. Zhou, A. M., Tewari, P. C., Bluestein, B. I., Caldwell, G. W. and Larsen, F. L.: Multiple forms of prostate-specific antigen in serum: Differences in immunorecognition by monoclonal and polyclonal assays. Clin. Chem., **39:** 2483, 1993.
9. Stamey, T. A.: Second Stanford conference on international standardization of prostate-specific antigen immunoassays: September 1 and 2, 1994. Urology, **45:** 173, 1995.
10. Laemmli, U. K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, **227:** 680, 1970.
11. Schagger, H. and von Jagow, G.: Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal. Biochem., **166:** 368, 1987.
12. Svendsen, L., Blombäck, B., Blombäck, M. and Olsson, P. I.: Synthetic chromogenic substrates for determination of trypsin, thrombin and thrombin-like enzymes. Thromb. Res., **1:** 267, 1972.
13. Stamey, T. A., Teplow, D. B. and Graves, H. C. B.: Identity of PSA purified from seminal fluid by different methods: comparison by amino acid analysis and assigned extinction coefficients. Prostate, **27:** 198, 1995.
14. Schaller, J., Akiyama, K., Tsuda, R., Hara, M., Marti, T. and Rickli, E. E.: Isolation, characterization and amino-acid sequence of γ-seminoprotein, a glycoprotein from human seminal plasma. Eur. J. Biochem., **170:** 111, 1987.
15. Wang, M. C., Valenzuela, L. A., Murphy, G. P. and Chu, T. M.: Purification of a human prostate specific antigen. Invest. Urol., **17:** 159, 1979.
16. Watt, K. W. K., Lee, P. J., M'Timkulu, T., Chan, W.-P. and Loor, R.: Human prostate-specific antigen: structural and functional similarity with serine proteases. Proc. Natl. Acad. Sci., **83:** 3166, 1986.
17. Lilja, H.: A kallikrein-like serine protease in prostatic fluid cleaves the predominant seminal vesicle protein. J. Clin. Invest., **76:** 1899, 1985.
18. Rusciano, D., Berardi, A., Ceccarini, C. and Terrana, B.: Concomitant purification of prostatic carcinoma tumor markers from human seminal fluid under nondenaturing conditions. Clin. Chem., **34:** 2528, 1988.
19. Mikolajczyk, S. D., Grauer, L. S., Millar, L. S., Hill, T. M., Kumar, A., Rittenhouse, H. G., Wolfert, R. L. and Saedi, M. S.: A precusor form of PSA (pPSA) is a component of the free PSA in prostate cancer serum. Urology, **50:** 710, 1997.
20. Stenman, U. H., Leinonen, J., Alfthan, H., Rannikko, S., Tuhkanen, K. and Alfthan, O.: A complex between prostate-specific antigen and α_1-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, 1991.
21. Leinonent, J., Zhang, W. M. and Stenman, U. H.: Complex formation between PSA isoforms and protease inhibitors. J. Urol., **155:** 1099, 1996.
22. Lilja, H., Christensson, A., Dahlén, U., Matikainen, M-T., Nilsson, O., Pettersson, K. and Lövgren, T.: Prostate specific antigen in human serum occurs predominantly in complex with α_1-antichymotrypsin. Clin. Chem., **37:** 1618, 1991.
23. Noldus, J., Chen, Z. and Stamey, T. A.: Isolation and characterization of free form prostate specific antigen (f-PSA) in sera of men with prostate cancer. J. Urol., **158:** 1606, 1997.