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Thiophilic-interaction chromatography of enzymatically active tissue prostate-specific antigen (T-PSA) and its modulation by zinc ions☆

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Abstract

Prostate-specific antigen (PSA) is a serine protease secreted both by normal prostate glandular epithelial cells and prostate cancer cells. We explored "thiophilic-interaction chromatography" (TIC) to isolate tissue prostate-specific antigen (T-PSA) from fresh human prostate cancer tissue harvested by radical prostatectomy for the purpose to characterize T-PSA for its enzymatic activity and sensitivity to zinc ions. We have shown, for the first time, that T-PSA has strong affinity for the thiophilic gel (T-gel). The average recovery of T-PSA from T-gel is over 87%. The presence of PSA in the column eluate was confirmed by ELISA and SDS/PAGE. Western blot developed with monoclonal antibody to PSA revealed that T-PSA was predominantly in the "free" form having a molecular weight of 33 kDa. Furthermore, T-PSA was found to be enzymatically active. T-PSA was found to be less enzymatically active as compared to seminal plasma PSA. The inhibition of enzymatic activity of both f-PSA and T-PSA over a wide range of concentrations of Zn^{2+} ions (10 nM to 50 μM) was comparable. In contrast, the enzymatic activity of chymotrypsin, another serine-protease, was affected differently. At higher concentrations of Zn^{2+} (10 μM and higher) the enzymatic activity of chymotrypsin was inhibited, whereas, at lower concentrations of Zn^{2+} (5 μM and lower), the enzymatic activity was enhanced.

Keywords: Prostate cancer; Prostate-specific antigen; T-PSA; Thiophilic-interaction chromatography; Zinc

1. Introduction

Prostate-specific antigen (PSA) is a member of the tissue kallikreine family of serine proteases [1]. PSA is a 33-kDa glycoprotein with chymotrypsin like activity that is produced primarily by the human prostatic epithelium and secreted into the seminal fluid [2,3]. The only well-recognized physiological function of PSA is the digestion of the seminogelins and fibronectin present in seminal coagulum, liquefying the seminal clot shortly after ejaculation [4]. In addition, PSA is a widely utilized serum biomarker for the diagnosis and management of prostate cancer [5]. In the seminal plasma, the majority of PSA is present as free PSA (f-PSA), and is enzymatically active. Approximately, 10% of seminal plasma PSA is complexed with protein C inhibitor (PSA-PCI) [6,7]. In contrast, the majority of PSA in serum is bound to serine protease inhibitors, including α-1-antichymotrypsin (PSA-ACT), α-2-macroglobulin (PSA-A_2M), and α-1-antitrypsin (PSA-AT) [8]. f-PSA accounts for only 5–10% of total PSA in the serum [9]. The major portion of prostatic T-PSA is f-PSA, whereas, the complexed PSA forms are <2% of the total PSA [10]. The physiological relevance of T-PSA, and its value in prostate cancer progression or management, is unclear. However, available evidence suggests that PSA is down-regulated in prostate cancer tissue and, thereby, T-PSA concentrations are lower in cancerous than in non-cancerous parts of the prostate [11].

Abbreviations: PSA, Prostate-specific antigen; f-PSA, free-PSA; T-PSA, tissue-PSA; TIC, Thiophilic Interaction Chromatography; T-gel, Thiophilic-gel; ELISA, Enzyme-linked immunosorbant assay; SDS/PAGE, sodium dodecylsulfate/polyacrylamide gel electrophoresis; PSA-ACT, PSA complexed with alpha-1 antichymotrypsin; PSA-A_2M, PSA complexed with alpha-2 macroglobulin; PSA-PCI, PSA complexed with protein C inhibitor; PSA-AT, PSA complexed with anti-trypsin.

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"Thiophilic-interaction chromatography" (TIC) technique was introduced originally by Porath and his colleagues for the isolation of immunoglobulins [12]. TIC has been used successfully for characterization of human transferrin [13], Alzheimer's beta-amyloid peptides [14] and for purification of immunoglobulins from chicken sera [15]. This chromatographic step is based upon salt-promoted adsorption of proteins to the resin, where the binding of proteins to a sulfone and thio-ether-containing hetero-aliphatic ligand takes place mainly through accessible tryptophan and/or phenylalanine residues. The desorption of bound proteins is achieved when the salt concentration is reduced [12]. In our earlier studies, we have shown that PSA and PSA complexes have strong affinity for different thiophilic gels (T-gels), and that T-gel affinity can be successfully applied for the purification of PSA and PSA-complexes from biological fluids, including serum and seminal plasma [16,17]. We have shown that f-PSA purified from seminal plasma by T-gel chromatography is enzymatically active [18]. In the present study, for the first time, the use of TIC technique has been applied for the isolation and subsequent characterization of T-PSA from freshly harvested human prostate cancer tissue. TIC was selected for isolation of T-PSA because we earlier have shown that T-gel has strong affinity for all molecular forms [free and complexed forms] of PSA [16,18]. Taking this approach, we were able to show that T-PSA, isolated from human prostate tumor tissue contains only the "free" form of PSA and that this PSA is enzymatically active. The enzymatic activity of T-PSA isolated from prostate tumor tissue is lower than enzymatic activity of f-PSA isolated from seminal plasma.

It is known that, Zn^{2+} concentrations that is abundantly present in prostate gland, varies significantly in normal and malignant human prostate tissue [19,20]. Zinc is also known to have a significant influence upon enzymatic activity of serine proteases that includes PSA [21,22]. The f-PSA isolated from seminal plasma and T-PSA isolated from prostate tissue facilitated studying the effect of zinc, and other essential trace elements, on the enzymatic activity of PSA. For comparative purposes, chymotrypsin, another serine protease, was used as a control. The substrate used for determination of enzymatic activity of PSA is highly specific for this serine protease [23].

2. Experimental

2.1. Seminal plasma

Based upon a protocol approved by the Institutional Review Board at Roswell Park Cancer Institute, large pools of leftover seminal fluid were obtained from the Infertility and IVF Medical Associates of Western New York after they were finished with all of their needs. The seminal fluid was spun at 10,000 × *g* for 20 min to remove cellular debris and the supernatant dialyzed overnight against 15 mM sodium phosphate buffer containing 0.15 M sodium chloride, pH 7.0. After dialysis, the seminal plasma was spun again at 10,000 × *g* for 20 min and the supernatant containing the PSA was frozen in small aliquots at −70 °C until used. None of the samples were frozen and thawed more than once.

2.2. Tissue samples

De-identified human prostate cancer tissue samples were obtained from the Tissue Procurement Core Resource at Roswell Park Cancer Institute under approval of the Institutional Review Board. Small pieces of tissue were dissected immediately after removal of the prostate and immediately stored in liquid nitrogen until analysis.

2.3. Preparation of tissue extracts

Fifty milligrams of frozen prostate cancer tissue samples were cut into small pieces and homogenized in a Wheaton glass homogenizer in 500 μl of extraction buffer that contained 0.05 M Tris–HCl, pH 7.4, 0.001 M EDTA, 0.001 M dithiothreitol, and 0.01 M NaCl. The homogenized tissue samples were sonicated three times, with 5 s of sonication and 30 s cooling between each sonication. The sonicated tissue extract was centrifuged at 100,000 × *g* for 30 min. The supernatant was removed and stored at −80 °C until analysis. The pellet was suspended in 500 μl of 0.05 M phosphate buffer, pH 7.4, 2 M NaCl, 0.002 M EDTA until analyzed for DNA contents.

2.4. DNA determination

DNA concentrations in the pellets of the prostate cancer tissue homogenates were determined using bis-benzimide, commonly known as Hoechst 33258 dye (H33258, Calbiochem, San Diego), according to the procedure described by Labarca and Paigen [24]. Tissue pellets were resuspended in 0.05 M phosphate buffer that contained 2.0 M sodium chloride and 2.0 mM EDTA, pH 7.4. Calf thymus DNA was used as a standard. Briefly, 5.0 μl of the dye (10 μg/ml) was added to 490 μl of 0.05 M phosphate buffer, 2.0 M NaCl, pH 7.4 and 5.0 μl of appropriately diluted tissue pellet test solution. Fluorescence was measured with a PerkinElmer LS-45 Luminescence Spectrometer (excitation 356 nm and emission 458 nm). Calf thymus DNA at concentration range 0.1–1.0 μg was used to prepare a standard curve.

2.5. Chromatographic ligands and other chemicals

Fractogel TA 650 (s) (T-gel) was purchased from EM Separation Science (Gibbstown, NJ, USA). Ultrogel AcA-54 was purchased from BioSepra, SA, Division of Ciphergen Biosystems (Fremont, CA, USA). Precast gradient gels and molecular weight standards for SDS/PAGE gel electrophoresis were obtained from Bio-Rad Labs (Hercules, CA, USA). Chemiluminescence reagents (ECL) were acquired from NEN Life Science Products (Boston, MA). α-chymotrypsin and BTEE were purchased from Sigma Chemical Company (St. Louis, MO, USA). All other chemicals and reagents used were of analytical grade, or of the highest purity available.

2.6. Thiophilic gel chromatography

T-gel slurry was packed in a BioRad Column (0.5 cm × 2.0 cm) and equilibrated with 25 mM HEPES buffer containing 1.0 M sodium sulfate, pH 7.0. Human prostate cancer tissue extract (500 μl) prepared in column equilibrating buffer was applied to the column. The column was washed with 20 ml of column equilibrating buffer at a flow rate of 12 ml/h. The bound cellular proteins, including the T-PSA, were eluted with 25 mM HEPES buffer, pH 7.0 that contained no salt. One-milliliter fractions were collected. The protein concentration was monitored by absorbance at 280 nm. The presence of PSA in the eluted fractions was detected by ELISA and SDS-PAGE/Western blot analysis using a monoclonal anti-PSA antibody.

2.7. SDS-PAGE and Western blot analysis

SDS-PAGE was performed under non-reducing conditions on 4–15% pre-cast gradient polyacrylamide gels using a Bio-Rad Mini-Protein II Unit (Bio-Rad, Hercules, CA) by the method of Laemmli [25]. Samples were mixed in a ratio of 1:1 (v/v) with sample buffer (125 mM Tris–HCl, pH 6.8) that contained 20% glycerol, 4% SDS, and 0.05% bromophenol blue. Ten microliters of each sample that contained 1.0 μg PSA was loaded into each well, and the separation gel run for 40 min at 200 V. Proteins were transferred electrophoretically to polyvinylidene difluoride (PVDF) membranes using mini trans-blot electrophoretic transfer cells (Bio-Rad, Hercules, CA). The transfer buffer contained 25 mM Tris, 192 mM glycine, and 20% methanol at pH 8.3. The transfer was completed in approximately 1 h at 100 V. After the transfer, the membrane was blocked with NAP-sure blocker (Geno Technology Inc., St. Louis, MO) with gentle agitation. The membrane was probed with a monoclonal anti-PSA antibody purchased from Dako (Carpinteria, CA, USA) in NAP-sure blocker for 1 h. Subsequently, the membrane was incubated for 45 min with secondary antibody, a peroxidase-conjugated goat anti-mouse IgG (Jackson Immuno Research Laboratories, West Grove, PA, USA), at 1:5000 dilution in 1.0% albumin in PBS containing 0.1% Tween 20. The membrane was washed and developed in chemiluminescent reagent (NEN, Boston, MA, USA) according to manufacturer's instructions. Blots were visualized by exposing the chemiluminescence reacted blot to X-ray film.

2.8. PSA measurement by ELISA

The quantitation of PSA in prostate tissue extracts and in various column eluates was carried out by sandwich ELISA. Polyclonal anti-PSA antibody (Dako, Carpinteria, CA, USA) was used as a "capture" antibody and a monoclonal anti-PSA antibody was used as the "detection" antibody. The assay was calibrated with purified PSA (Calbiochem) and a linear correlation was obtained in the range of 0.75–25 ng/ml PSA. Microtiter plates (Nunc-Immuno plate, Maxisorp. Nunc Inc., Naperville, IL, USA) were coated overnight with 50 μl/well of appropriately diluted capture antibody in PBS at 4 °C. The plate was washed three times with 400 μl of PBS that contained 0.01% Tween 20 (wash buffer) and was blocked with 200 μl of 2.0% BSA solution in PBS (block buffer) for 1 h. Fifty microliters of each test sample at appropriate dilutions were added to triplicate wells and incubated for 1 h. After incubation with the primary antibody, plates were washed three times with 400 μl of wash buffer, 50 μl of appropriately diluted detection antibody added to each well, and the plate incubated for 1 h. After washing each well three times with 400 μl of wash buffer, 50 μl of peroxidase-labelled IgG was added and incubated for 45 min. After incubation, the plate was again washed three times with 400 μl of wash buffer, 100 μl of 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate solution added to each well, and the plate incubated for 15–20 min avoiding direct light. The reaction was stopped by adding 50 μl of 2.0 N sulphuric acid and the color intensity was measured at 450 nm in an ELISA plate reader.

2.9. Separation and purification of f-PSA from seminal plasma

f-PSA was purified from seminal plasma by a two step procedure described previously [18]. Briefly, thiophilic-gel chromatography was used as a first step in the purification procedure. PSA and PSA-complexes are retained on this matrix, along with immunoglobulins. In the second step of the purification, f-PSA was separated from the PSA-complexes and immunoglobulins by size-exclusion chromatography on an AcA-54 column. Fractions containing f-PSA were collected, concentrated and filter sterilized. The purity of f-PSA was confirmed by 2D-gel electrophoresis.

2.10. Enzymatic activity of PSA

The enzymatic activity of f-PSA purified from seminal plasma, and T-PSA purified from prostate cancer tissue, was characterized by the procedure described by Denmeade et al. [23] using a highly "selective" fluorogenic substrate (Mu-His-Ser-Ser-Lys-Leu-Gln-AFC) obtained from Calbiochem (San Diego, CA, USA). The hydrolysis of this fluorogenic substrate was monitored on a LS 45 Luminescence Spectrometer (PerkinElmer) using the FL-Winlab® program. Assays were performed by mixing f-PSA from seminal plasma, or T-PSA from prostate cancer tissue, at a concentration of 5.7 nM with the fluorogenic substrate (38 μM) dissolved in assay buffer (50 mM Tris–HCl, pH 7.9 that contained 10 mM NaCl) to a final volume of 530 μl, and the mixture incubated at 25 °C. Enzymatic activity was determined by recording the linear increase of fluorescence for 20 min: excitation was set at 400 nm and emission at 505 nm. The blank was prepared containing the substrate in assay buffer. The details of the procedure are described elsewhere [18,26].

2.11. Modulation of enzymatic activity of f-PSA and T-PSA by Zn^{2+} ions

The effect of Zn^{2+} ions on the enzymatic activity of f-PSA from seminal plasma and T-PSA from prostate cancer tissue was evaluated by incubation of f-PSA or T-PSA (5.7 nM) with a range of concentrations of $ZnCl_2$ (0.001–50 μM) in assay

buffer [50 mM Tris–HCl, containing 10 mM NaCl, pH 7.9] for 10 min at 25 °C. Substrate concentration was constant (38 μM) in all reactions. In the presence of $ZnCl_2$, fluorescence readings were consistently lower, suggesting quenching of fluorescence. Therefore, $ZnCl_2$ blanks also were prepared. Enzymatic activity of any PSA preparations was determined by recording the initial linear increase of fluorescence for 20 min.

2.12. Effect of trace elements on the enzymatic activity of f-PSA

The effect of different essential trace elements on the enzymatic activity of f-PSA purified from seminal plasma was tested. The enzymatic activity of f-PSA (5.7 nM) in an assay buffer containing increasing concentrations (0.01–50 μM) of different trace elements was studied. In all the cases, the substrate concentration was kept constant at 38 μM. The different essential trace elements under investigation included copper, cadmium, nickel, boron, and selenium. Appropriate blanks were prepared and the residual enzymatic activity of f-PSA was determined by recording the linear increase of fluorescence for 20 min.

2.13. Assay of the enzymatic activity of chymotrypsin

The enzymatic activity of chymotrypsin was determined using *N*-α-Benzoyl-L-tyrosine ethyl ester (BTEE) as the substrate. Chymotrypsin readily acts upon amides and esters of susceptible amino acids, and hydrolyses this specific substrate into *N*-α-Benzoyl-L-tyrosine and ethanol. The reaction velocity was determined according to the procedure of Hummel [27] by measuring the increase in absorbance at 256 nm using a 6405 UV–vis-Spectrophotometer (Jenway Limited, Felsted, Dunmow, Essex, UK). Assays were performed by mixing α-chymotrypsin (26 nM) with increasing concentrations of BTEE (100–600 μM) dissolved in assay buffer (80 mM Tris–HCl, pH 7.8 that contained 100 mM $CaCl_2$), to a final volume of 3.0 ml, and incubating the reaction mixture at 25 °C. The increase in absorbance at 256 nm was recorded for 10 min, and ΔA_{256} nm/min was calculated using the maximum linear rate. Chymotrypsin (26 nM) was incubated with a range of concentrations of $ZnCl_2$ (0.3–300 μM) in assay buffer at 25 °C for 10 min. After incubation with $ZnCl_2$, chymotrypsin specific substrate BTEE (100–600 μM) was added to the reaction mixture separately. Then the residual enzymatic activity of chymotrypsin was determined by recording the increase in absorbance at 256 nm for 10 min, and ΔA_{256} nm/min was calculated using the maximum linear rate.

3. Results

3.1. Chromatography of human prostate cancer tissue homogenate on Fractogel TA650s

Human prostate cancer tissue extract that contained 13.59 μg of T-PSA was reconstituted in 25 mM HEPES, 1 M sodium sulfate, pH 7.0, and applied to a column (0.5 cm × 2 cm) packed with T-gel slurry as described in Section 2.6. The column was washed with 20 ml of column equilibrating buffer. One-milliliter fractions were collected. Approximately, 90% of the proteins applied to the column had no affinity for the column matrix and were eluted in the breakthrough region. T-PSA was bound tightly to the matrix in the presence of high salt (1.0 M sodium sulfate). After the unbound proteins were washed from the column, the bound proteins were eluted with 25 mM HEPES buffer that contained no sodium sulfate. Fig. 1 presents a chromatogram of the separation of a prostate tissue homogenate on Fractogel TA650s. The presence of protein in the column fractions was monitored by absorbance at 280 nm. PSA in the breakthrough/wash fractions and in eluted fractions was monitored by a sandwich ELISA assay. Approximately, 85% of the T-PSA applied to the column was recovered after T-gel chromatography.

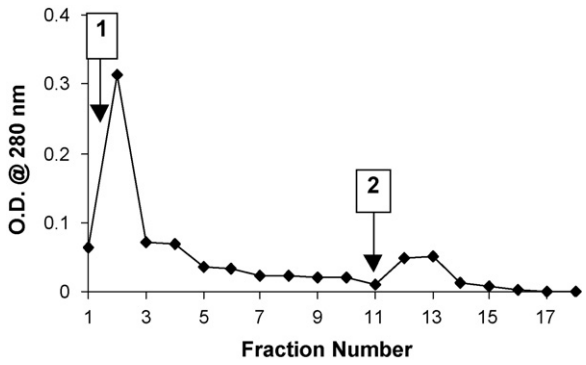

Fig. 1. Chromatography of human prostate cancer tissue homogenate on Fractogel TA650s: 500 μl of tissue homogenate was applied to the column equilibrated with 25 mM Hepes buffer containing 1 M sodium sulfate, pH 7.0 (1). After washing the column with column equilibrating buffer, the bound proteins containing PSA were eluted with 25 mM Hepes buffer, pH 7.0 (2). The presence of protein in various column eluates was monitored by their absorbance at 280 nM and presence of PSA was monitored by sandwich ELISA.

Tumor tissue homogenates were prepared individually from 50 mg of prostate cancer tissue from nine separate tumor specimens and were processed through Fractogel TA 650s columns. PSA levels in various column fractions were monitored by sandwich ELISA. DNA content in the tissue pellets was determined using the Hoechst 33258-dye method. The level of T-PSA in prostate cancer tissue was expressed as nanogram PSA/μg DNA. The levels of T-PSA in the tumor specimens ranged from 0.8 to 80 ng PSA/μg DNA. Table 1 shows that the percent recovery of

Table 1
Recovery of human prostate cancer tissue-PSA (T-PSA) after Fractogel TA650s chromatography

Prostate cancer tissue #	T-PSA applied (μg)	T-PSA recovered (μg)	Recovery (%)
1	13.59	11.59	85.3
2	14.16	13.00	91.8
3	4.20	3.70	88.0
4	0.14	0.12	85.0
5	3.67	3.24	88.2
6	3.18	2.75	86.4
7	14.70	12.40	84.3
8	86.90	78.20	89.9
9	3.22	2.82	87.6

T-PSA from the nine tumor tissue samples ranged from 85 to 91.8%, with an average recovery of 87%.

3.2. SDS-PAGE/Western blot analysis of T-PSA

T-PSA from prostate cancer tissue, after processing through T-gel chromatography step, was further analyzed under non-reducing conditions by SDS/PAGE Western blot analysis using monoclonal antibody to PSA. The objective here was to determine what molecular form(s) of PSA are present in the prostate tumor tissue extract. Since PSA is a low abundant protein, it is essential that majority of proteins are removed from prostate tumor tissue extract before different molecular forms (free and complexed) of PSA can be detected by SDS/PAGE Western blot analysis. T-gel chromatography made it possible to accomplish this goal. Sera from prostate cancer patients are known to have different molecular forms of PSA including free and complexed PSA. Both T-gel processed prostate cancer patient serum and f-PSA acquired from a commercial source (Calbiochem, Santiago, CA, USA) were included in SDS/PAGE Western blot analysis as controls. The results are shown in Fig. 2. The serum from prostate cancer patient, as expected, has both f-PSA and complexed PSA. Monoclonal antibody to PSA used here is known to recognize all known molecular forms of PSA. The identity of a complex-PSA can be further confirmed by using specific antibody to a specific serine protease inhibitor. However, prostate tumor tissue extract gave a single band corresponding f-PSA. This band was identical to commercially acquired f-PSA that has a molecular weight of 33 kDa. No additional PSA band was ever seen in any of T-PSA preparations analyzed by SDS/PAGE.

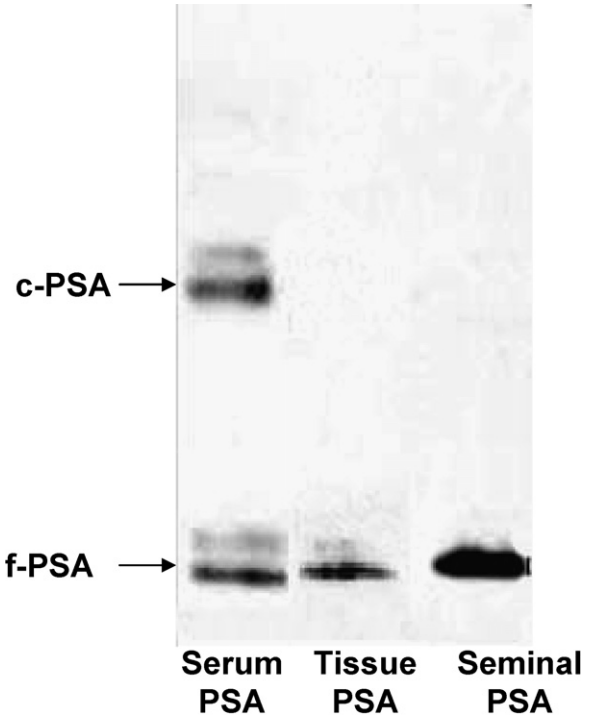

Fig. 2. SDS/PAGE Western blot analysis of PSA under non-reducing conditions in prostate cancer patient serum (left lane); in prostate cancer tissue (middle lane) and in seminal plasma (right lane) as detected by monoclonal anti-PSA antibody.

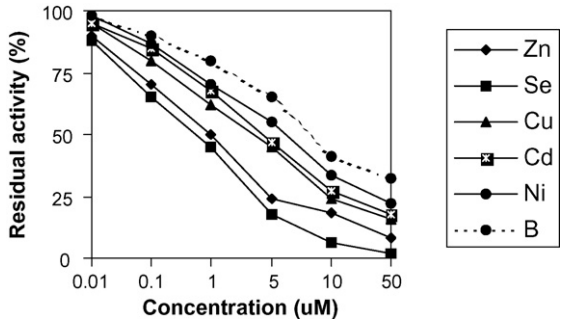

Fig. 3. Effect of different essential trace elements on the enzymatic activity of f-PSA. The enzymatic activity of f-PSA (100 ng) in a total assay volume 530 μl of buffer containing increasing concentrations of different essential trace elements was monitored. The f-PSA-mediated release of AFC from the highly specific fluorogenic substrate (38 μM) is expressed as a percentage of maximal f-PSA activity in the absence of any trace elements.

This strongly suggests that prostate tumor tissue has largely f-PSA.

3.3. Effect of Zn^{2+} and other trace elements on the enzymatic activity of f-PSA

We reported previously that f-PSA isolated from seminal plasma by T-gel chromatography had a significant level of enzymatic activity [18]. Human prostate tissue and seminal plasma are known to have the highest concentration of zinc in the human body [28]. Therefore, it is of relevance to know the effect of zinc on the enzymatic activity of PSA. Fig. 3 demonstrates that Zn^{2+} inhibited enzymatic activity of purified f-PSA in a concentration dependent manner. Zinc at a 1.0 μM concentration gave a 50% inhibition of enzymatic activity of f-PSA. Among the trace elements tested, at equimolar concentrations, selenium gave the maximal inhibition of PSA enzymatic activity while Boron was least effective in inhibiting f-PSA enzymatic activity. Under these reaction conditions, a 1578-fold molar excess of boron to f-PSA was required to achieve 50% inhibition. Table 2 shows the concentration for each of the trace elements that are required for 50% inhibition of enzymatic activity of f-PSA isolated from seminal plasma. Over 11-fold and 9-fold higher concentrations of boron were needed compared to selenium and zinc, respectively, to produce a 50% inhibition of f-PSA activity.

Table 2
Effect of different essential trace elements on the enzymatic activity of f-PSA

Trace elements	Concentration (μM)[a]	Molar excess (fold control)[b]
Zinc	1.0	175
Selenium	0.8	140
Copper	4.0	701
Cadmium	5.0	877
Nickel	7.5	1315
Boron	9.0	1578

[a] Concentration of the essential trace elements at which the enzymatic activity of f-PSA is half-maximal (50%).

[b] Number of fold molar excess of essential trace elements compared to f-PSA concentration (5.7 nM).

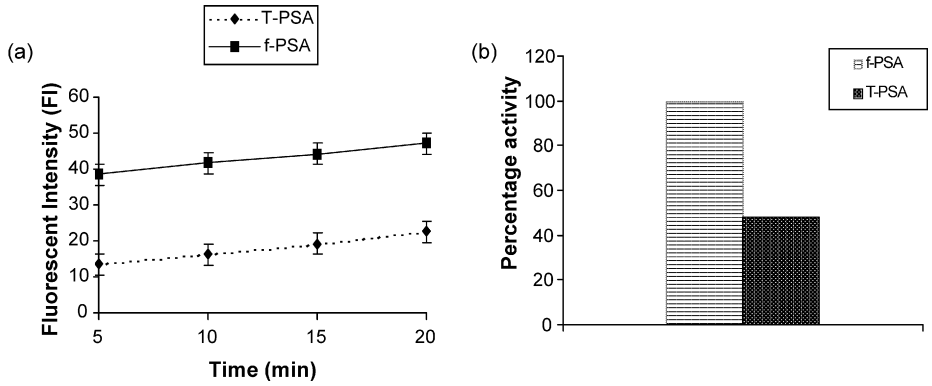

Fig. 4. Enzymatic activity of prostate cancer tissue PSA (T-PSA). (a) The reaction mixture contained 20 μg of highly specific chromogenic substrate and 100 ng of T-PSA or f-PSA in a 530 μl of total assay volume. The enzymatic activity of T-PSA was found to be reduced compared with that of f-PSA activity when all other conditions were identical. The activity was determined by recording the initial linear increase of fluorescence for 20 min in both the cases. Data presented are the mean ± S.D. values from three different experiments. (b) A bar diagram showing the relative reduction (approximately 50%) in the enzymatic activity of T-PSA compared with that of f-PSA when all other assay conditions are kept constant.

3.4. Enzymatic activity of T-PSA

T-PSA separated from prostate cancer tissues was characterized for enzymatic activity under comparable incubation conditions. The concentration of T-PSA in the assay mixture was maintained at 5.7 nM, comparable to that of f-PSA in the previous experiments. Assays were performed by mixing T-PSA with the fluorogenic substrate (38 μM) dissolved in assay buffer (50 mM Tris–HCl, pH 7.9 that contained 10 mM NaCl) to a final volume of 530 μl, and the incubation performed for 20 min at 25 °C. Fig. 4(a) and (b) show the difference in enzyme activity between f-PSA and T-PSA when all experimental conditions were kept constant. The enzymatic activity of T-PSA was found to be reduced 50% per 100 ng PSA protein compared to that of f-PSA.

3.5. Effect of Zn^{2+} on the enzymatic activity of T-PSA

T-PSA (5.7 nM) was incubated with a range of concentrations of $ZnCl_2$ (0.001–50 μM) in assay buffer (50 mM Tris–HCl, pH 7.9 that contained 10 mM NaCl) at 25 °C for 10 min. Substrate concentration was kept constant (38 μM) in all reactions. The residual enzymatic activity of T-PSA was determined by recording the initial linear increase of fluorescence for 20 min. Increasing Zn^{2+} concentrations increased the level of inhibition of enzymatic activity of T-PSA. Unlike f-PSA, Zn^{2+} was the most effective inhibitor of T-PSA (Fig. 5). At 0.2 μM of Zn^{2+}, T-PSA enzymatic activity was inhibited by 50%, and T-PSA enzymatic activity was completely inhibited at concentrations of zinc above 10 μM. Zinc is more effective in inhibiting enzymatic activity of T-PSA as compared to seminal plasma f-PSA (Figs. 3 and 5): 1 μM of zinc inhibited 50% of f-PSA activity, but only 0.2 μM zinc was required for similar inhibition of T-PSA enzymatic activity.

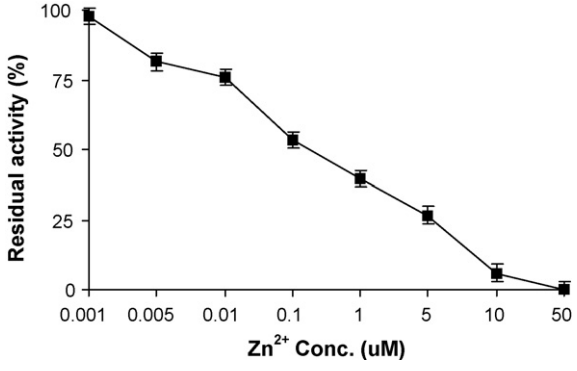

Fig. 5. Effect of Zn^{2+} concentration on the enzymatic activity of prostate cancer tissue PSA (T-PSA). The enzymatic activity of T-PSA (100 ng) in a buffer containing increasing concentrations of Zn^{2+} was studied. Residual activity is expressed based on the intensity of AFC released from the highly PSA specific fluorogenic substrate. Data presented are the mean ± S.D. values from three different experiment.

3.6. Effect of Zn^{2+} on the enzymatic activity of chymotrypsin

The primary and three-dimensional structure of PSA indicates that PSA is a serine protease with chymotrypsin-like activity [29]. The effect of zinc ions on the enzymatic activity of chymotrypsin was studied by the procedure described in Section 2.13. At lower concentrations (from 300 nM to 3.3 μM), Zn^{2+} ions increased the activity of chymotrypsin up to 12%. There was no increase in activation observed below 300 nM. Furthermore, when the concentration of Zn^{2+} ions was increased to above 3.3 μM, the enzymatic activity of chymotrypsin decreased considerably, depending upon the final concentration of $ZnCl_2$. Fig. 6(a) and (b) demonstrate the modulating effect of Zn^{2+} ions on the enzymatic activity of chymotrypsin.

4. Discussion

Many attempts have been made to isolate PSA and its molecular forms from seminal plasma using various chromatographic procedures [30,31]. Our group has developed a simple two-step procedure that provides homogeneous, pure preparations of human PSA [18]. The procedure is applicable for isolation of PSA from all biological fluids including serum, seminal plasma and tissue culture medium, etc. In the present study, we utilized

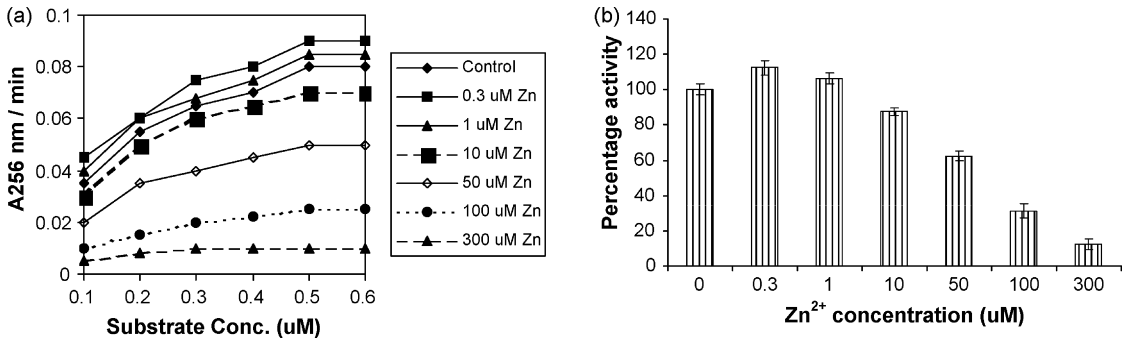

Fig. 6. Effect of Zn^{2+} concentration on the enzymatic activity of chymotrypsin. (a) α-Chymotrypsin (26 nM) was incubated with a range of concentrations of $ZnCl_2$ (0.3–300 μM) in an assay buffer (80 mM Tris–HCl, 100 mM $CaCl_2$, pH 7.8) at 25 °C for 10 min. The assays were performed by mixing increasing concentrations of BTEE substrate (100–600 μM) to the reaction mixture. The residual enzymatic activity of chymotrypsin was determined by recording the increase in absorbance at 256 nm for 5 min and ΔA256 nm/min was obtained using the maximum linear rate. A graph was plotted by taking ΔA256 nm/min values in *y*-axis and concentration of BTEE in *x*-axis. (b) Graph showing the effect of different dosages of Zn^{2+} upon the enzymatic activity of chymotrypsin. Data presented are the mean ± S.D. values from three different experiments.

the T-gel procedure to isolate PSA from human prostate cancer tissues. Tissue PSA (T-PSA) also had a strong affinity for T-gel, was largely uncomplexed and demonstrated enzymatic activity.

We evaluated T-PSA levels in prostate cancer tissues from nine independent patients. Each sample was processed through T-gel chromatography, and the recovered PSA was analyzed subsequently by ELISA and Western blot analysis. The average recovery of T-PSA from tumor tissue is 87.4% (Fig. 1 and Table 1). Western blot analysis revealed a single band at M_r 33,000, demonstrating that T-PSA is largely free, or uncomplexed with inhibitors (Fig. 2). This finding supports an earlier study [32] that reported that intracellular PSA exists largely in the "free" form.

T-PSA isolated from prostate cancer tissue had a reduced enzymatic activity (50%) compared to PSA isolated from seminal plasma (Fig. 4(a) and (b)) when evaluated at equivalent concentrations. There may be several reasons for the reduced activity of T-PSA. Firstly, it is possible that T-PSA loses some of its enzymatic activity during the process of extraction from tissue. Secondly, the two respective sources of PSA may have different levels of zinc, and the level of zinc may alter enzymatic activity. Finally, PSA isolated from seminal plasma and from prostate cancer tissue may have different specific activity. The f-PSA obtained from seminal plasma has a component that has no intrinsic enzymatic activity [18]. Two peptide sequences were identified for the PSA from seminal plasma; one corresponding to full length PSA and the other to an N-1 sequence that is missing the N-terminal isoleucine residue. The two sequences comprise roughly 70% and 30%, respectively, of the total f-PSA from seminal plasma. The full-length sequence is known to be enzymatically active, whereas, the N-1 sequence has no enzymatic activity. T-PSA isolated from prostate cancer tissue may also have both enzymatically active and inactive components, and their proportions may vary relative to PSA from seminal plasma.

The scientific literature on the physiology and pathobiology of PSA remains unclear. Several reports have suggested that PSA may act as a tumor suppressor, a negative regulator of cell growth, and an apoptotic molecule [33–35], whereas, others suggest that PSA may, through its serine protease activity, promote tumor progression and metastasis [36–38]. The precise relationship between the reported functions of PSA in prostate cancer cells *in vitro* and the biological role of PSA as a serine protease in human prostate cancer *in vivo* is unknown.

Zinc is known to play an important role in the development and normal functioning of the prostate, and normal prostate tissues from healthy individuals accumulate the highest zinc levels in the body; the zinc concentration in the prostate and prostatic fluid is around 9.0 mM [39]. Zinc ions inhibit PSA at micromolar levels, indicating that Zn^{2+} is a tightly binding inhibitor of PSA activity [21], and that the endogenous level of Zn^{2+} in the human prostate could suppress the invasion and metastasis of prostate cancer cells through regulation of the proteolytic activity of PSA [22]. We evaluated in vitro the kinetics of inhibition of PSA purified from human seminal plasma and from human prostate cancer tissue extracts by comparing the Zn^{2+}-mediated modulation of PSA activity. In addition, the effect of different essential trace elements, including copper, cadmium, nickel, selenium and boron on the enzymatic activity of this purified f-PSA also was evaluated. The invasive ability of LNCaP cells was slightly suppressed by Cu^{2+} ions [22]. Cadmium is a suspected prostatic carcinogen and can induce malignant transformation of non-tumorigenic, human prostatic epithelial cells *in vitro* [40]. Like cadmium, nickel also is a suspected prostatic carcinogen. Specific interest in selenium as a chemopreventive agent for prostate cancer has arisen recently [41]. Lastly, PSA activity is inhibited *in vitro* by boron and boronated compounds [26].

Several previous reports addressed the effect of these trace elements on the enzymatic activity of PSA obtained either commercially or isolated in crude form [21,22,26,41]. This study, based upon the availability of large quantities of high quality f-PSA from seminal plasma, confirmed that PSA was inhibited by different essential trace elements, and demonstrated that the most effective inhibitors are zinc and selenium (Fig. 3). Furthermore, we observed that f-PSA and T-PSA are differentially sensitive to inhibition by zinc. A 5-fold higher concentration of Zn^{2+} (1 μM) is needed to achieve a 50% inhibition of enzyme

activity of purified f-PSA compared to the concentration of Zn^{2+} needed to inhibit the enzymatic activity of T-PSA (0.2 μM) to half-maximal levels (Figs. 3 and 5). The limited availability of prostate tissue samples, and the difficulty in recovery of sufficient quantities of T-PSA, prevented us from evaluating the effect on enzymatic activity of T-PSA of the full panel of trace metals. Binding of Zn^{2+} to the amino acid residues of the catalytic triad of PSA (His 91, His 101 or His 233) could distort the architecture through minor movements of His 101 which is immediately adjacent to the active site residue, Asp 102 [42]. Furthermore, the binding of a Zn^{2+} ion in this part of the molecule may modify the conformation of the loop 95, blocking the entrance to the specificity pocket [43].

Zinc ions act as effectors for serine proteases, such as chymotrypsin. Serine proteases can be either activated [44] or inhibited [45], depending upon the Zn^{2+} concentrations. Fig. 6(a) and (b) demonstrate that Zn^{2+} ions affect the enzymatic activity of chymotrypsin in a 'dose-dependent manner'. At lower concentrations, Zn^{2+} ions increased the activity of chymotrypsin. When the concentration of Zn^{2+} ions was increased, the enzymatic activity of chymotrypsin decreased proportionately with respect to the concentration of $ZnCl_2$. In contrast, low concentrations of zinc ions did not increase enzymatic activity of PSA, but rather, low concentrations of zinc inhibited the enzymatic activity of T-PSA. The marked inhibition observed at these low concentrations suggested that the endogenous levels of Zn^{2+} present in the prostate could be important in the regulation of PSA enzymatic activity *in vivo*. These studies also indicated an absence of a dose-dependent modification of PSA enzyme activity by Zn^{2+}. This difference from chymotrypsin may be attributed to several reasons. PSA is a member of the tissue kallikrein family of acidic serine proteases, whereas, chymotrypsin is a member of the basic serine protease family. In addition, the substrate specificity of PSA is distinct from that of all other serine proteases. PSA cleaves proteins mainly on the C-terminus of tyrosine, histidine, leucine, and glutamine residues [23,29].

In conclusion, the data presented in this report suggest that T-gel affinity can be employed for the isolation of T-PSA from prostate tissues, and the availability of a large quantity of T-PSA would facilitate greatly the development of efficient inhibitors/modulators for *in vitro* and *in vivo* evaluation. Our findings on the regulation of PSA activity by low concentrations of zinc could greatly facilitate further research focused on understanding the importance of PSA enzymatic activity at both intracellular and extracellular levels in prostate homeostasis.

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