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Two step procedure for purification of enzymatically active prostate-specific antigen from seminal plasma

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Abstract

The role of prostate-specific antigen (PSA) during the onset of prostate cancer and subsequent tumor growth and metastasis is not well understood. We have developed a simple two step procedure, based on principles of hydrophobic charge-induction chromatography and molecular size chromatography to provide pure free-PSA (f-PSA) preparation that is free from all other known PSA complexes as well as human kallikrein 2 (hK2). The overall recovery of f-PSA is 72%. The isolated f-PSA consists of three known isoforms that corresponds to p*I* of 6.2, 6.4 and 7.2. f-PSA is enzymatically active and its enzymatic activity can be effectively neutralized by a serine protease inhibitor.

Keywords: Prostate cancer; Prostate-specific antigen; PSA; Serine protease; Thiophilic-interaction chromatography; PSA purification

1. Introduction

Prostate-specific antigen (PSA), a serine protease and member of tissue kallikrein family of proteins, is a 33 kDa glycoprotein that has chymotrypsin and trypsin like activity [1]. PSA is one of the most abundant protein, largely produced by the human prostatic epithelium at a concentration ranging from 0.5 to 3 mg/ml [2,3]. The known physiological function of PSA is to degrade seminal vesicle proteins that are important components of semen coagulum [4,5].

PSA is a well known tumor marker and its serum values have been very useful in early diagnosis and in the management of prostate cancer [4]. Besides prostate cancer, serum PSA values are elevated in other tumor types, including breast and ovarian cancers [6]. In prostate cancer, the excess PSA spills over into circulation where it exists either as free PSA (f-PSA) or as PSA that complexes with various serine protease inhibitors [7,8]. In serum, PSA complexes with α-1 antichymotrypsin (PSA–ACT), α-2-macroglobulin (PSA–A_2M), α-1-antitrypsin (PSA–AT) [9,10] and protein C inhibitor (PSA–PCI) [11]. The physiological relevance of any of these circulating forms of PSA with regard to prostate cancer diagnosis or management is not known. Also, currently there is no methodology available that can selectively identify and quantitate these molecular forms in the serum; particularly from the point of view of clinical diagnosis. In the serum, a small fraction of total PSA remains as f-PSA. On the contrary, the majority of total PSA in the seminal plasma is f-PSA and the predominant complex is PSA–PCI [11]. Nearly 70% of f-PSA in the seminal plasma is enzymatically active [12]. In our earlier studies, we have shown that PSA and PSA-complexes have strong affinity for different Thiophilic gels (T-gel) and that T-gel affinity can be explored for the purification of f-PSA and PSA-complexes from various biological fluids including serum and seminal plasma [13,14].

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In recent literature, PSA has been documented as an anti-angiogenic molecule and an inducer of apoptosis [15,16]. PSA has been shown to inhibit endothelial cell proliferation, migration and invasion [16]. PSA also inhibits endothelial cell response to FGF2 and VEGF that are known to stimulate angiogenesis [16]. Transfection of PSA c-DNA in PC-3 prostate carcinoma cells prolongs their doubling time, reduces their tumorigenicity and metastasis in nude mice [17]. These observations collectively support the idea that PSA may have a role in prostate tumor growth and metastasis. In order to critically assess the role of PSA as a "biomarker" or as a "therapeutic modality", it is critical to have a source of PSA that is of high quality, well characterized and enzymatically active. Although, cloning of PSA has been reported in recent literature [18], it is critical that PSA from a natural source should be used initially for assessing its physiological functions. We have developed a simple two-step procedure that allows isolation of enzymatically active f-PSA from seminal plasma with high over all recoveries. The availability of high quality active f-PSA will facilitate evaluation of the physiological role(s) of PSA in prostate tumor cell proliferation, migration and metastasis.

2. Experimental

2.1. Seminal plasma

Large pools of the 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 Institutional Review Board at Roswell Park Cancer institute had approved this study. The seminal fluid was initially centrifuged at $10{,}000 \times g$ for 20 min to remove the cellular debris and the supernatant (seminal plasma) was dialyzed overnight against 15 mM of phosphate buffer, pH 7.0 containing 0.15 M sodium chloride. At the end of dialysis, seminal plasma was centrifuged again and the supernatant was aliquoted and frozen at $-70\,^{\circ}C$ until used. None of the samples were thawed more than once. Seminal plasma contained on an average of 0.3–0.5 mg of PSA per milliliter.

2.2. 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 Cipher-gen Biosystems (Fremont, CA, USA). f-PSA was obtained from Calbiochem (San Diego, CA, USA). Gradient gels and molecular weight standards for gel electrophoresis were obtained from BioRad Labs, (Hercules, CA, USA). Chemiluminescence reagents (ECL) were acquired from NEN Life Science Products (Boston, MA, USA). All other chemicals and reagents used were of analytical grade, or otherwise of highest purity available.

2.3. Antibodies

Mono- and polyclonal anti-PSA antibodies and polyclonal anti-α-antichymotrypsin antibody were purchased from Dako (Carpinteria, CA, USA). Peroxidase conjugated goat-anti mouse and goat anti-rabbit antibodies were purchased from Jackson Immuno Research Laboratories (West Grove, PA, USA). Polyclonal anti-protein C-inhibitor antibody was obtained from Cedar Lane Laboratories (Hornby, Ont., Canada).

2.4. Protein determination

Protein concentrations were determined using the bicinchoninic acid (BCA) protein assay procedure using 96-well microtiter plates [19]. The reagents for this assay were purchased from Pierce (Rockford, IL, USA). Bovine serum albumin was used as a standard.

2.5. Thioiphilic gel chromatography

Twenty-five millimeters of T-gel slurry was packed in a BioRad column (2.5 cm × 10 cm) and equilibrated with 25 mM Hepes buffer containing 1 M sodium sulfate, pH 7.0. Fifty milliliters of seminal plasma dialyzed against column equilibrating buffer was applied to the column. The column was washed with column buffer at a flow rate of 20 ml/h. The bound proteins were eluted with 25 mM Hepes buffer, pH 7.0 containing no sodium sulfate. Two milliliter fractions were collected. The presence of PSA in various column fractions was detected by SDS/PAGE Western blot analysis using anti-PSA antibody.

2.6. Concentration of seminal plasma proteins

The T-gel column eluates containing PSA (f-PSA, PSA–PCI and PSA–ACT) were pooled and concentrated using a stir-cell (Amicon Corp., Lexington, MA, USA) equipped with ultrafiltration membrane, YM-10 with 10,000 MW cut-off (Millipore Corp., Bradford, MA, USA) kept at 4 °C. The concentrated material containing free and complexed PSA and containing on an average 2–3 mg/ml PSA.

2.7. Ultrogel® AcA-54 column chromatography

A column (100 cm × 2.5 cm, Amersham Biosciences, Piscataway, NJ, USA) was packed with Ultrogel AcA-54 (bead size 60–140 μM), having a fractionation range of 5000–70,000 kDa, as per instructions from the manufacturer. The column was equilibrated with 10 mM sodium acetate buffer containing 0.15 M sodium chloride, pH 5.6. The column was calibrated periodically with known molecular weight gel-filtration standards from Bio-Rad (Bio-Rad Labs, Hercules, CA, USA). For each run, 3 ml of concentrated PSA containing semino protein solution after thiophilic

gel chromatography, was applied under column buffer. The column was developed at room temperature at the rate of 16 ml/h and 4 ml fractions were collected. Each fraction was monitored for the presence of protein by BCA protein assay procedure. The presence of PSA was monitored either by SDS/PAGE/Western-blot analysis or by ELISA. In subsequent runs, PSA was monitored in various column eluates ranging in molecular weight between 25 and 40 kDa by Western-blot analysis. The final quantitation of PSA was based upon double determined ELISA using poly and monoclonal anti-PSA antibodies.

2.8. SDS/PAGE and Western-blot analysis

SDS/PAGE was performed by the method of Laemmli [20]. Electrophorsis was carried out under non-reducing conditions through 4–15% gradient polyacrylamide gel using Bio-Rad-Protein II unit (Bio-Rad). Samples were mixed in a ratio of 1:1 (v/v) with sample buffer (125 mM Tris–HCl, pH 6.8) containing 20% glycerol, 4% SDS, and 0.05% bromophenol blue. Ten microliters of each sample containing 15–20 μg protein was loaded into each well and run for 40 min at 200 V. The proteins were then electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes using mini trans-blot electrophoretic transfer cells (Bio-Rad). The transfer buffer contained 25 mM Tris, 192 mM glycine, and 20% methanol at pH 8.3. The transfer was performed for 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 membranes were probed with appropriate antibodies in NAP-sure blocker for 1 h. Secondary antibody, peroxidase conjugated goat anti-mouse IgG at 1:5000 dilution in 1% albumin in PBS containing 0.1% Tween 20 was incubated with the membrane for 45 min. The membranes were 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.

2.9. PSA measurement by ELISA

Determination of PSA in various chromatographic fractions or in various preparations of f-PSA was carried out by sandwich ELISA. In this assay, polyclonal anti-PSA antibody was used as "capture" antibody and a monoclonal anti-PSA antibody was used as "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 plates were washed three times with 200 μl of PBS containing 0.01% Tween 20 and blocked with 200 μl of 2% BSA solution in PBS for 1 h. Fifty microliters of each test sample at appropriate dilution was added to triplicate wells and incubated for 1 h. For detection, 50 μl of appropriately diluted detection antibody was added to each well and the plate was incubated for 1 h. The plates were exposed to peroxidase labeled secondary antibody (50 μl) for 45 min. After washing the plate, a substrate solution containing 0.02% H_2O_2, and 0.4 μg/ml of *O*-phenlydiamine in 0.2 M citrate buffer, pH 5.0 was added to each well. The reaction was stopped by adding 50 μl of 2N H_2SO_4 and the intensity of color was measured at 450 nm in an ELISA plate reader.

2.10. 2-D gel electrophoresis

Two-dimentional gel electrophoresis was carried out using Novex system and their reagents (Invitrogen, Carlsbad, CA). Thirty microliters of purified f-PSA (1.5 μg/μl) was mixed with 30 μl of Novex 2X IEF sample buffer, pH 3–10 and applied in two lanes of Novex pre-cast IEF gels (pH 3–10). Five microliters of Bio-Rad IEF standards, pH 4.45–9.6 were also loaded in one of the lanes. Isoelectric focusing was performed using Novex Xcell II Mini-cell system at constant voltage of 100 V for 1 h, followed by 200 V for 1 h and 500 V for last 30 min. The gels were stained with Novex Colloidal Blue Staining Kit and destained with several changes in double distilled water. Lanes containing samples were cut out of the gel and electrophoresed in the second dimension in Novex 8–16%, 1.5 mm thick Tris–glycine gel (Invitrogen, Carlsbad, CA, USA) using Novex Xcell II Mini-Cell system as per instructions from the manufacturer. Novex SeeBlue prestained protein standards were loaded into one of the lanes. Gels were electrophoresed at the constant voltage of 125 V until dye front reached the bottom of the gel. Following electrophoresis in the second dimension, Western blotting, immunostaining and chemilluminescent detection was carried out as described above using anti-PSA antibody.

2.11. Enzymatic activity of purified f-PSA

The purified f-PSA was characterized for its enzymatic activity using a fluorogenic substrate (Mu–His–Ser–Ser–Lys–Leu–Gln–AFC) obtained from Calbiochem (San-Diago, CA, USA). The test is based on the hydrolysis of this fluorogenic substrate that is specific for PSA enzymatic activity [21] The activity was determined from the initial linear increase of fluorescence and were expressed as micromoles of AMC released per min based on comparison to a standard curve of the fluorescence of known amounts of the substrate. The fluorescence was measured using Aminco-Bowman Spectrophotoflurometer (American Instrument Co., Silver Spring, MD, USA). The excitation was set at 400 nm and emission at 505 nm. The details of the procedure are described elsewhere [21].

2.12. Amino-terminal sequencing of f-PSA protein

f-PSA purified from seminal plasma was prepared for sequencing, following SDS/PAGE and blotting on to

polyvinylidene difluoride membrane as described by Matsudaira [22]. The protein band was subjected to at least 10 cycles of Edman degradation and parathyroid hormone amino acid detection was performed on an Applied Biosystems 494 cLC protein sequencer equipped with an on-line HPLC system. The procedure was carried out by the W.M. Keck Foundation Biotechnology Resource Laboratory, Yale University, New Haven, CT, USA.

3. Results

3.1. Chromatography of seminal plasma proteins on Fractogel TA650s

Dialyzed seminal plasma was applied to a column packed with, T-gel slurry as per the details provided in Section 2. The results are shown in Fig. 1. Nearly 90% of the proteins applied to the column had no affinity for this ligand and they appeared in the breakthrough region. After column was adequately washed, the bound proteins were eluted with 25 mM Hepes buffer containing no sodium sulfate. The presence of PSA, both free and complexed forms (PSA–PCI and PSA–ACT) was detected by SDS/PAGE/Western-blot analysis using monoclonal anti-PSA antibody (Fig. 1, inserts A and B). The majority of PSA in seminal plasma is f-PSA. The identity of PSA–PCI and PSA–ACT was subsequently confirmed respectively by using anti-PCI and -ACT antibodies (data not shown). This step in purification effectively separated the majority of seminal proteins from PSA and at the same time retaining all molecular forms of PSA known to be present in the seminal plasma (Fig. 1, insert B). The experiment was repeated several times and results were essentially reproducible. The average recovery of total PSA in this step in purification was 92%.

3.2. Molecular size chromatography (AcA-54 gel) of T-gel processed seminal proteins

Further separation of f-PSA from other contaminating proteins including immunoglobulins and PSA-complexes was achieved by molecular size chromatography step. Seminal proteins, after T-gel chromatography, containing all molecular forms of PSA were concentrated to an average of 2–3 mg/ml PSA protein and applied to column packed with AcA-54 gel (fractionation range between 5 and 70 kDa). The column was equilibrated with 10 mM sodium acetate buffer containing 0.15 M sodium chloride, pH 5.6. An average of 90% protein appeared in the exclusion range. The presence of PSA in various column fractions was monitored by SDS/PAGE/Western-blot analysis and probed with anti-PSA antibody. The results are shown in Fig. 2. The starting material for “molecular size chromatography” contained f-PSA, PSA-complexes and high molecular weight proteins

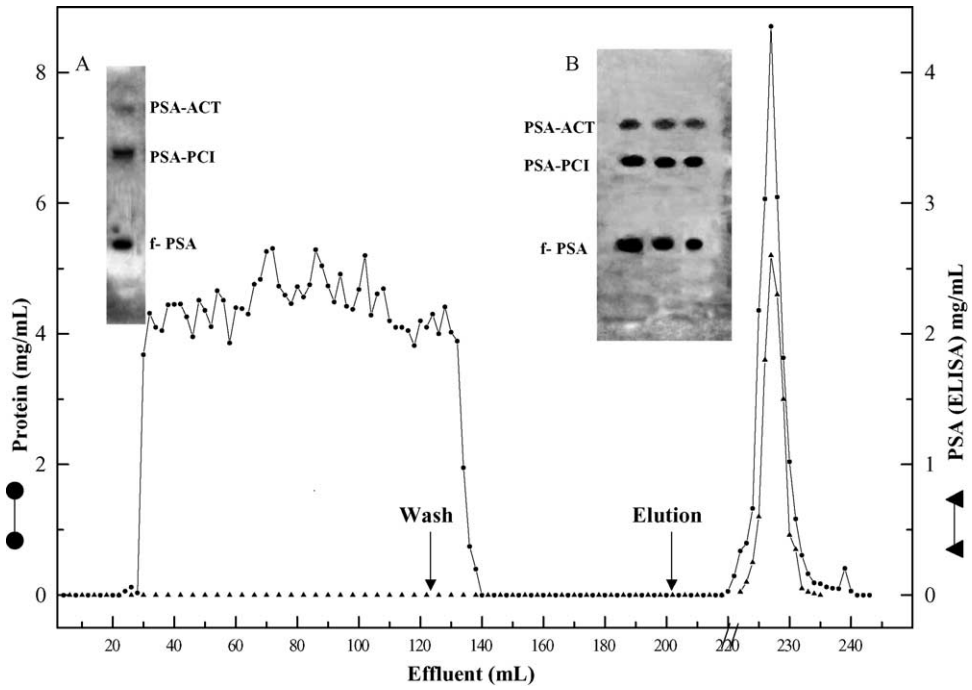

Fig. 1. Chromatography of seminal plasma on Fractogel TA650s. The column was equilibrated with 25 mM Hepes buffer containing 1 M sodium sulfate, pH 7.0. Seminal plasma was dialyzed against column equilibrating buffer and applied to the column. The column was washed and the bound proteins were eluted with 25 mM Hepes buffer. The protein concentration was measured by BCA method and PSA was monitored by either SDS/PAGE Western-blot analysis using monoclonal anti-PSA antibody or by double determined ELISA. Insert: (A) PSA isoforms (f-PSA, PSA–PCI and PSA–ACT) present in the starting material. The presence of PSA–PCI and PSA–ACT was subsequently confirmed by monoclonal antibodies to PCI and ACT. (B) All three PSA isoforms were recovered in the column eluates.

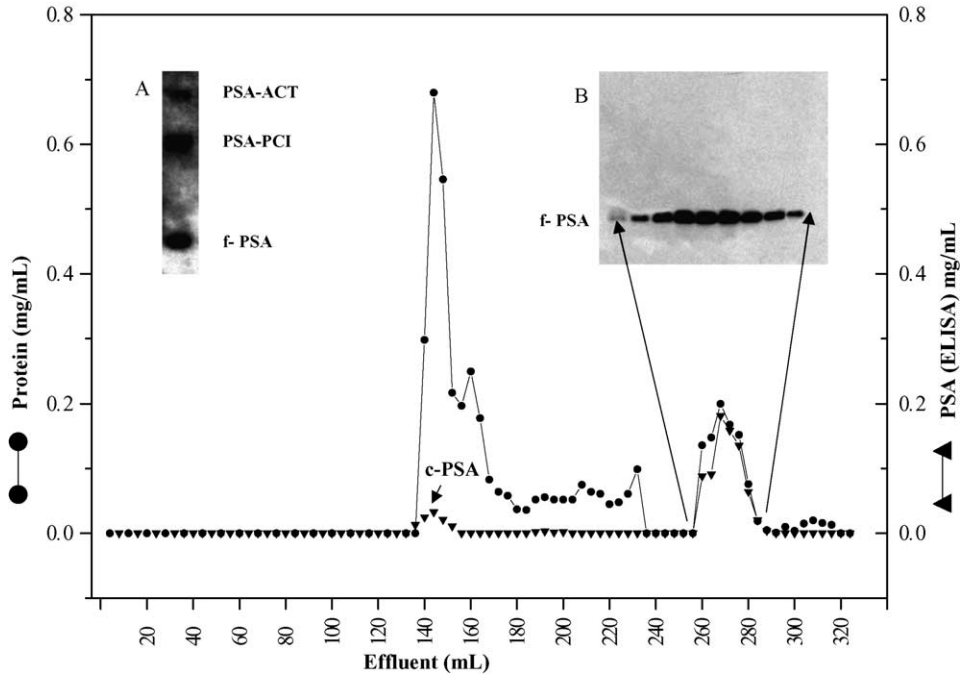

Fig. 2. Chromatography of T-gel processed seminal proteins on Ultrogel AcA-54 column. The column was equilibrated with 10 mM sodium acetate buffer containing 0.15 M sodium chloride, pH 5.6. The concentrated seminal proteins, after T-gel chromatography and containing f-PSA, PSA–PCI and PSA–ACT and other proteins were applied to the column. The column was developed at the flow rate of 16 ml/h and 4 ml fractions were collected. The protein was monitored by the BCA protein assay procedure. PSA presence was monitored either by SDS/PAGE Western-blot analysis using anti-PSA antibody or quantitated by the double determined ELISA using anti-PSA antibodies. Insert: (A) f-PSA, PSA–PCI and PSA–ACT in the starting material. (B) f-PSA.

including immunoglobulins (Fig. 2, insert A). Immunoglobulins and other high molecular weight proteins appeared in the column breakthrough region. During this step both f-PSA and complexed PSA were also distinctly separated (Fig. 2, inserts). Fractions containing f-PSA were pooled, concentrated and filter sterilized. PSA concentration was adjusted to 1 mg/ml protein and aliquots were frozen at −70 °C. The average recovery of PSA was 82%. The proportion of complexed PSA (c-PSA) largely containing PSA–PCI and PSA–ACT was < 10% of total PSA applied [23]. The f-PSA preparations were further confirmed to be free from PSA–PCI and PSA–ACT complexes by subjecting f-PSA preparations to SDS/PAGE Western-blot analysis and staining with anti-PCI and anti-ACT antibodies. f-PSA preparations were subsequently evaluated for its purity and enzymatic activity.

3.3. PSA recovery

The data in Table 1 shows the percent recovery of PSA from three different experiments. It includes PSA recoveries at each step in the purification procedure and also the overall PSA recoveries at the end of the process. The average recovery of PSA after T-gel chromatography is 92% and after AcA-54 molecular size chromatography is 82%. The overall recovery of f-PSA at the end of these two steps in purification varied with different seminal plasma pools and varied in between 64 and 78% range. This percent recovery of PSA is considerably higher than what has thus far been reported in the literature. The PSA recovered after T-gel chromatography contained both f-PSA and complexed-PSA (PSA–PCI, PSA–ACT). However, at the end of second step in purification, the PSA recovery as shown in Table 1 represents only f-PSA and does not include complexed-PSA that is usually about 10% of the total PSA. Complexed-PSA; being higher in molecular weight, appeared much earlier in elution profile (Fig. 2, c-PSA peak).

3.4. Characterization of purified f-PSA

3.4.1. Purity of f-PSA as determined by 2-D gel electrophoresis

The high-resolution power of two-dimentional gel electrophoresis (2-D gel) was used to test the purity of f-PSA preparation. The purified f-PSA was concentrated to 1 mg/ml protein and stored either at −70 °C or in few cases lyophilized. The f-PSA was subjected to 2-D gel electrophoresis as per the details described above in Section 2. One gel was stained with silver nitrate to show protein bands and another gel was developed with monoclonal antibody to PSA. The results are shown in Fig. 3. Protein staining has shown three bands, all in the region of 33 kDa and with respective p*I* of 6.2, 6.4 and 7.2. These three bands represent already known isoforms of PSA [11,24]. No additional protein bands were seen any where on the gel (Fig. 3a). Fig. 3b represents the gel, ran identically to Fig. 3a, and stained with monoclonal anti-PSA antibody. The PSA antibody also stained

Table 1
PSA recovery at each step in purification

Hydrophobic charge induction chromatography			Molecular size chromatography			
PSA[a] applied (mg)	PSA recovered (mg)[a]	Recovery (%)	PSA applied (mg)[a]	f-PSA recovered (mg)	Recovery (%)	Overall recovery (%)
25.5	24.45	95.9	23.44	20.10	82.2	78.8
17.8	16.3	91.4	14.9	11.3	75.8	63.5
22.68	20.41	90.1	19.56	16.82	86.7	74.2

[a] Includes free and complex forms.

identical three spots corresponding to p*I* of 6.2, 6.4 and 7.2. They all represent f-PSA.

3.4.2. Enzymatic activity of f-PSA

The majority of f-PSA present in the seminal plasma (~70%) is known to be enzymatically active [25]. We used a highly specific substrate (Mu–His–Ser–Ser–Lys–Leu–Gln–AFC) to determine if f-PSA preparation has enzymatic activity [21]. The data presented in Fig. 4 shows the enzymatic activity of f-PSA. PSA was able to hydrolyze this substrate in a dose dependent manner (Fig. 4). With increasing concentration of f-PSA, the amount of AFC released is increased proportionately over a period of time. The enzymatic activity of f-PSA obtained by our method consists of an average of 211 units as compared to 169 units of commercially acquired enzymatically active PSA (Calbiochem, Cat. #539834, Lot #B40577). One unit of enzymatic activity is defined as the amount of enzyme that cleaves 1 μmol of substrate/min/ml. In order to ascertain that the enzymatic activity of PSA is indeed specific to this substrate, we attempted to block PSA enzymatic activity by a serine protease inhibitor, aprotinin. When PSA was first exposed to Aprotinin prior to the substrate, its enzymatic activity was completely eliminated (Fig. 4).

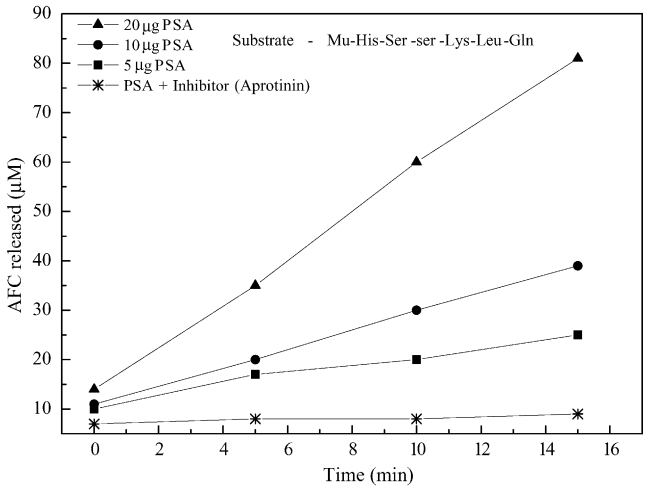

Fig. 4. Enzymatic activity of f-PSA. The reaction mixture contained 10 μl of 20 mM concentration of highly specific chromogenic substrate and varying concentrations of f-PSA (5, 10 and 20 μg) in total of 500 μl of 50 mM Tris. HCl, pH 7.9 buffer. The amount of AFC released over a period of time was monitored using Aminco-Bowman Spectrophotometer. There is a dose-dependent increase in amount of AFC released by increasing concentration of f-PSA. The enzymatic activity of f-PSA was completely blocked when f-PSA was exposed to a serine protease inhibitor, Aprotinin before being exposed to the substrate.

3.4.3. Amino-terminal sequencing of f-PSA

A sample of f-PSA preparation was sent to W.M. Keck Foundation Biotechnology Resources Laboratory, Yale University for amino-terminal sequencing. The protein was subjected to at least 10 cycles of amino acid sequence analysis. The results are shown in Table 2. There were two major sequences and both were in agreement with NCBI data base for PSA. A full length sequence that has isoleucine as the N-terminal residue and constituted 70% of total f-PSA and another as N – 1 sequence and starting with valine at the

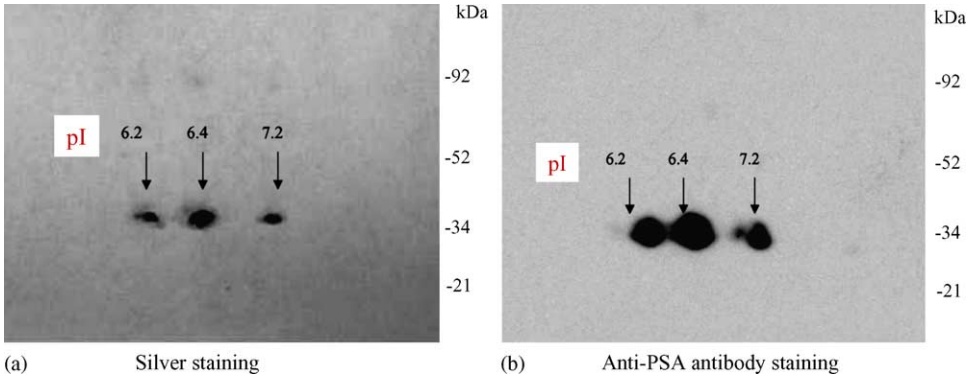

Fig. 3. 2-D gel electrophoresis of f-PSA. The blots were developed either with silver stain (a) or by chemiluminescent agent after probing the membrane with anti-PSA antibody (b). Three distinct spots were seen corresponding to p*I* 6.2, 6.4 and 7.2. The identical spots were illuminated in both gels. Beside PSA isoforms, no other proteins were detected in gel stained with silver nitrate.

Table 2
Amino-terminal sequence of f-PSA(seminal plasma)

[25]Ile–Val–Gly–Gly–Trp–Glu–Cys–Glu–Lys–His–full length
[26]Val–Gly–Gly–Trp–Glu–Cys–X–Lys–His–Ser–N − 1

N-terminus. This represent 30% of f-PSA. Occasionally, a third sequence (<5%) starting [170]Lys–Leu–Gly–X–Val–Asp was seen. This was also confirmed to be PSA sequence with molecular weight of <10 kDa.

4. Discussion

We have developed a simple two-step procedure to obtain pure preparation of f-PSA from seminal plasma. The main highlights of our procedure are: (1) the simplicity of the procedure, (2) the significantly higher recoveries of f-PSA (Table 1), the purified f-PSA preparation is free from any known PSA complexes (Fig. 3) and (3) f-PSA preparation is enzymatically active (Fig. 4). Our procedure is milder and avoids any denaturation conditions. The use of thiophilic-gel chromatography as a first step in this purification procedure allows handling of large volume of seminal plasma as this ligand retains <10% of total proteins that are applied. The seminal plasma proteins that are retained on this ligand includes PSA, all known PSA-complexes and some high molecular weight proteins including immunoglobulins. This ligand has high capacity (10–15 mg protein/ml adsorbent) and can be used, both in a column chromatography procedure or in a batch adsorption procedure. The PSA complexes and other high molecular weight proteins including immunoglobulins were further separated from f-PSA during the second step, based on molecular size chromatography, on AcA54 column. The column was developed in a buffer at pH 5.6 to avoid autocleavage of PSA during purification. The overall recovery of purified f-PSA range between 64 and 78% of total PSA present in the starting material. This represents a significant improvement in overall PSA recovery that has been reported earlier. The average recoveries as reported in the literature range between 7 and 30% of the total PSA [2,11,26]. In majority of these cases, the PSA was characterized either as a single band in SDS/PAGE or by amino terminal sequencing and not by its enzymatic activity. The immunological characterization of PSA was based upon mono- and polyclonal antibodies to PSA in SDS/PAGE/Western-blot analysis. The purity of f-PSA preparation was initially confirmed by 2-D gel electrophoresis. The corresponding gels were either exposed to silver stain to visualize protein bands or to monoclonal anti-PSA antibody to visualize immunoreactive PSA bands. It is known that PSA exist in different isoforms [11,24]. The variability of different isoforms may be due to differences in either the primary structure of the protein or in the pattern of glycosylation or both. We have identified three isoforms of f-PSA corresponding to p*I* 6.2, 6.4 and 7.2. These three isoforms were consistently seen in different preparations of f-PSA purified from seminal plasma.

The more definitive identity of PSA protein was based on N-terminal sequence data (Table 2). Two sequences were identified; one corresponding to full length PSA and another to N − 1 with missing isoleucine. The two sequences were roughly 70 and 30%, respectively of the total PSA. The full length sequence is known to be enzymatically active whereas, N − 1 sequence has no enzymatic activity [15]. The third PSA sequence (<10 kDa) representing <5% of total f-PSA was seen occasionally. Evidently this sequence was not present initially in our purified f-PSA preparations. It is primarily because the second step in purification involved molecular sizing which effectively must separate the low molecular weight PSA sequence from the full length sequence. Perhaps, this sequence was generated as an artifact due to storage and/or handling of the f-PSA. The PSA gene (hK3) is a member of human tissue kallikrein gene family that consist of at least three well characterized members [27]. Seminal plasma is known to have both human kallikrein-2 (hK2) and human kallikrein-3 (hK3) proteins. Because of extensive sequence homology between these two kallikrein, it is conceivable that our f-PSA preparation may have traces of hK2. However, the probability of hK2 presence in our f-PSA preparation is next to nothing. Firstly, in the seminal plasma hK2 levels are 80–500-fold lower than hK3. In seminal plasma, PSA (hK3) levels are 0.5–3.0 mg/ml [2,3], whereas average hK2 level is 6 μg/ml [28]. It is also known that nearly all hK2 present in the seminal plasma complexes with PCI rather immediately [29] and the complex is much higher in molecular weight than f-PSA and will effectively be eliminated at the second step in our purification procedure.

Purified f-PSA has been shown to be enzymatically active (Fig. 4). The enzymatic activity is distinctly dose dependent. This enzymatic activity was effectively blocked by an inhibitor of serine protease (Fig. 4). It is known that enzymatic activity is associated with complete sequence and that lack of isoleucine at the start of N-terminus effectively destroys its enzymatic activity [30]. In our preparations, 70% of f-PSA is enzymatically active and this is in agreement to earlier reports [25].

Despite the importance of PSA as a surrogate marker for early detection of prostate cancer, relatively few reports have addressed the issue of its physiological functions and its relation to the pathogenesis and progression of prostate cancer. The most widely accepted physiological function of PSA is to liquefy the seminal clot. More recently, attention has been drawn to PSA as regulatory molecule in prostate tumor growth and metastasis. We have earlier shown that prostate cancer cell lines that vary in their metastatic potential have differential expression of growth factors that control tumor growth and metastasis [31]. Human prostate cancer cell line, PC3M, is known to be highly metastatic when inoculated into nude mice [32]. These cells have higher expression of growth factors like VEGF, IL-8, TGF-β2, ICAM1 and MMP9 that are known to promote tumor growth as compared to LNCaP cells that are poorly metastatic and are known to produces low levels of PSA in culture [31]. On the contrary PC3M cells

being highly metastatic have low level of HuIFN-γ expression [31]. HuIFN-γ is known to be a tumor suppressor. In subsequent studies, we have shown that PC3M cells treated with our purified f-PSA significantly down-regulates VEGF, pim1 oncogene, uPAR and up-regulates HuIFN-γ expression [33]. These observations provide a strong evidence that f-PSA has a role in overall tumor growth and metastasis.

The availability of large quantities of high quality PSA from natural source like seminal plasma would greatly facilitate further research in this direction and in the development of novel inhibitors/modulators for in vitro and in vivo evaluation.

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References

[1] D.A. Armbruster, Clin. Chem. 39 (1993) 181.
[2] M.C. Wang, L.A. Valenzuela, G.P. Murphy, T.M. Chu, Oncology 39 (1982) 1.
[3] G.F. Sensabaugh, E.T. Blake, J. Urol. 144 (1990) 1523.
[4] E.P. Diamandis, Trends Endocrinol. Metab. 9 (1998) 310.
[5] H. Lilja, J. Oldbring, G. Rannevik, C.B. Laurell, J. Clin. Invest. 80 (1987) 281.
[6] E. Kucera, C. Kainz, C. Tempfer, R. Zeillinger, H. Koelbl, G. Sliutz, Anticancer Res. 17 (1997) 4735.
[7] A. Christensson, C.B. Laurell, H. Lilja, Eur. J. Biochem. 194 (1990) 755.
[8] W.M. Zhang, P. Finne, J. Leinonen, S. Vesalainen, S. Nordling, U.H. Stenman, Clin. Chem. 45 (1999) 814.
[9] A. Christensson, H. Lilja, Eur. J. Biochem. 220 (1994) 45.
[10] H. Lilja, World J. Urol. 11 (1993) 188.
[11] W.M. Zhang, J. Leinonen, N. Kalkkinen, B. Dowell, U.H. Stenman, Clin. Chem. 41 (1995) 1567.
[12] H. Lilja, Br. J. Urol. 79 (Suppl. 1) (1997) 44.
[13] K.C. Chadha, E. Kawinski, E. Sulkowski, J. Chromatogr. B Biomed. Sci. Appl. 754 (2001) 521.
[14] E. Kawinski, E. Levine, K. Chadha, Prostate 50 (2002) 145.
[15] A.H. Fortier, J.W. Holaday, H. Liang, C. Dey, D.K. Grella, J. Holland-Linn, H. Vu, S.M. Plum, B.J. Nelson, Prostate 56 (2003) 212.
[16] A.H. Fortier, B.J. Nelson, D.K. Grella, J.W. Holaday, J. Natl. Cancer. Inst. 91 (1999) 1635.
[17] M.D.J.P. Balbay, N. Ilansa, S. Williams, D. McConkey, I.J. Fidler, et al., Proc. Am. Assoc. Cancer Res. 49 (1999) 225 (Abstract).
[18] L.L. Habeck, R.M. Belagaje, G.W. Becker, J.E. Hale, L.M. Churgay, M. Ulmer, X.Y. Yang, K.A. Shackelford, J.M. Richardson, M.G. Johnson, L.G. Mendelsohn, Prostate 46 (2001) 298.
[19] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, D.C. Klenk, Anal. Biochem. 150 (1985) 76.
[20] U.K. Laemmli, Nature 227 (1970) 680.
[21] S.R. Denmeade, W. Lou, J. Lovgren, J. Malm, H. Lilja, J.T. Isaacs, Cancer Res. 57 (1997) 4924.
[22] P. Matsudaira, J. Biol. Chem. 262 (1987) 10035.
[23] F. Espana, J. Sanchez-Cuenca, C.D. Vera, A. Estelles, J. Gilabert, J. Lab. Clin. Med. 122 (1993) 711.
[24] J.T. Wu, B.W. Lyons, G.H. Liu, L.L. Wu, J. Clin. Lab. Anal. 12 (1998) 6.
[25] U. Stenman, P. Finne, W. Zhang, J. Leinonen, Urology 56 (2000) 893.
[26] H.C. Graves, M. Kamarei, T.A. Stamey, J. Urol. 144 (1990) 1510.
[27] G.M. Yousef, E.P. Diamandis, Endocr. Rev. 22 (2001) 184.
[28] J. Lovgren, C. Valtonen-Andre, K. Marsal, H. Lilja, A. Lundwall, J. Androl. 20 (1999) 348.
[29] D. Deperthes, G. Frenette, M. Brillard-Bourdet, L. Bourgeois, F. Gauthier, R.R. Tremblay, J.Y. Dube, J. Androl. 17 (1996) 659.
[30] R. Kurkela, A. Herrala, P. Henttu, H. Nai, P. Vihko, Biotechnology (NY) 13 (1995) 1230.
[31] R. Aalinkeel, M.P. Nair, G. Sufrin, S.D. Mahajan, K.C. Chadha, R.P. Chawda, S.A. Schwartz, Cancer Res. 64 (2004) 5311.
[32] C.A. Pettaway, S. Pathak, G. Greene, E. Ramirez, M.R. Wilson, J.J. Killion, I.J. Fidler, Clin. Cancer. Res. 2 (1996) 1627.
[33] B. Bindukumar, S.A. Schwartz, M.P.N. Nair, R. Aalinkeel, K.C. Chadha, Neoplasia, in press.