

A Simplified and Reproducible Two-Step Method for the Purification of Prostate-Specific Antigen

Eskandar Kamali Sarvestani¹, Abdolaziz Khezri², Mahmoud Vessal³ and Abbas Ghaderi^{1*}

¹Dept. of Immunology, ²Urology and ³Biochemistry, Medical School, Shiraz University of Medical Sciences, Shiraz, Iran

ABSTRACT

Prostate-specific antigen (PSA) was purified to homogeneity from human seminal plasma by ion-exchange chromatography on a CM-Sephadex C-50 and by gel filtration on a Sephacryl S-200 column. A single 33-kDa protein band appeared in SDS-PAGE. High pressure liquid chromatography (HPLC) of the purified protein produced a single peak, while isoelectric focusing demonstrated the presence of five different isoforms of this protein. The immunoreactivity of the purified PSA was checked by Western blotting. This simple two-step method can be used for a large-scale preparation of the purified PSA for the clinical tests and also for further investigative studies on the biological properties of this protein. *Iran. Monied J. 3: 87-91, 1999*

Keywords: Prostate-specific antigen, purification, semen, chromatography

INTRODUCTION

Prostate-specific antigen (PSA) is a protease with a molecular weight of 33-34 kDa [1]. The molecule consists of approximately 92% protein and 8% carbohydrate [2]. PSA exists in at least five isoforms with isoelectric points (pI) ranging from 6.8 to 7.5 [2]. The different isoforms do not appear to be encoded by different genes but seem to result from different sialic acid content. PSA is the most frequently used marker in oncology. In fact, the high levels of the PSA in serum are strongly associated with prostate cancer [3]. In addition, PSA has different functions.

Originally, the main physiological function of the PSA was thought to be liquefaction of seminal coagulum [4]. However, PSA has been found to catalyze the hydrolysis of the other substrates such as insulin-like growth factor binding protein-3 (IGFBP-3) [5]. IGFBP-3 is a serum binding protein for insulin-like growth factor II (IGF-II), which is a growth factor for a variety of cells. Thus, the hydrolysis of the IGFBP-3 by PSA may modulate the levels of IGFs and consequently change the rates of cellular proliferation. PSA was shown to catalyze the degradation of extracellular matrix proteins such as fibronectin and laminin [6], thereby; it is implicated in the invasion of tissues by tumor cells. In order to perform a large-scale clinical tests and to investigate the functions of this

molecule, a constant supply of the purified PSA is required. This communication describes a simplified and efficient procedure for the purification of the various isoforms of this protein.

MATERIALS AND METHODS

Preparation of samples. Seminal plasma was obtained from apparently healthy donors referred to Nemazi hospital, Shiraz-Iran. Semen was allowed to liquefy and sperms were removed by centrifugation at 10,000 x g for 20 min. The supernatant fluid was stored at -20°C until used.

Protein determination and PSA assay. Total protein was measured by the method of Lowry *et al.* [7], using bovine serum albumin as a standard. The level of the PSA was determined using a commercial kit (PSA EIA, CanAg Diagnostics, Sweden). The detection limit of the assay was 0.1 µg/lit.

Purification of PSA. Pooled seminal plasma was dialyzed extensively against 0.1 M phosphate buffer (PB, pH 6.75). The dialyzed material was centrifuged (10,000 x g, 20 min) and the supernatant (20 ml) was applied to a 30 × 2.5 cm CM-Sephadex C-50 column pre-equilibrated with PB. The column was washed with PB and a large peak of contaminated proteins

*Corresponding Author: Dept. of Immunology, Medical School, Shiraz University of Medical Sciences, P.O. BOX 71345-1798, Shiraz, Iran. Tel/Fax: (98-71) 334589, E-mail: inununol@stuns.ac.ir

were eluted and discarded until the absorbance of the effluent reached under 0.2 Au. Then, the column was washed with a linear gradient of 0.0-0.5 M NaCl in 0.1 M PB. The fractions from the PSA containing peak were pooled and concentrated to a final volume of 2 ml using 70% ammonium sulfate. The concentrated fraction was dialyzed against phosphate buffered saline (PBS). After centrifugation (10,000 \times g, 20 min.), the supernatant was applied to a 16 \times 70 cm Sephacryl S-200 High Resolution column (Pharmacia Biotech, Uppsala, Sweden) pre-equilibrated with the same buffer. Proteins were eluted with the same PBS at a flow rate of 0.5 ml/min. Fractions containing the PSA were pooled, concentrated to a final volume of 2 ml using 70% ammonium sulfate and again dialyzed against PBS.

SDS-PAGE and Western blotting. The fractions from different steps of the PSA purification were analyzed by SDS-PAGE in a 10% gel according to the method described by Laemmli [8]. Fraction from each purification step was electrophoresed and detected by Coomassie Brilliant Blue or electroblotted onto nitrocellulose membranes. The membranes were blocked with 2.5% casein dissolved in PBS containing 0.05% Tween-20, and incubated for 2 h with the supernatant of hybridoma producing anti-PSA antibody or anti-gp63 as a negative control antibody. Anti-PSA monoclonal antibody was developed in our laboratory by the fusion of Immunized Balb/c splenocytes and SP2/O-Ag14 myeloma partner. After the cloning of produced hybridoma, the isotype was determined to be IgG1. Conjugated rabbit anti-mouse Ig (DAKO A/S, Denmark) in 1/1000 dilution was added to the blots. The antigen band was visualized by the addition of the substrate solution (40 mg diaminobenzidine + 7.5 μ l H₂O₂ in 100 ml PBS).

Isoelectric focusing. A 4% polyacrylamide gel with the thickness of 2 mm was employed for isoelectric focusing. The ampholine of pH range from 3.5 to 9.5 was also used. Electrode strips were soaked in 1 M H₃PO₄ (anode buffer) and 1 M NaOH (cathode buffer). Electrophoresis was run for 1 hr at 25 W with a maximum voltage.

High Pressure Liquid Chromatography (HPLC). HPLC was performed using a Waters 650E system. The analytical gel filtration column was a Protein pak-125 (7.8 \times 300 mm). The flow rate was 0.5 ml/min and the run was isocratic. The mobile phase was PBS (pH 7.2).

RESULTS

Purification of PSA. CM-Sephadex C-50 effectively retained PSA after extensive washing with PB. The elution of the PSA was achieved with a linear gradient of 0.0-0.5 M NaCl in the same buffer (Figure 1). Further purification of the protein was performed by gel filtration on Sephacryl S-200. The pooled CM-Sephadex C-50 fractions containing PSA were applied to a Sephacryl S-200 column and resolved into several protein peaks (Figure 2). PSA was found in fractions 155-170 ml. Table 1 outlines the results of typical purification steps.

Purity determination. Two methods were used to measure the purity of the PSA. In the first method, the product of different purification steps was subjected to the analytical polyacrylamide gel electrophoresis.

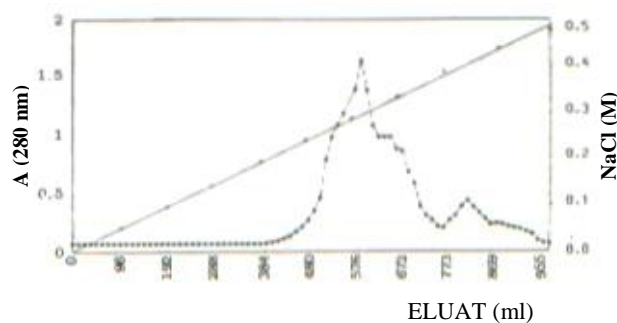


Fig. 1. CM-Sephadex column chromatogram of the seminal plasma. See the methods for the details of the experiment. (0) indicates initiation of linear salt gradient. PSA appeared in fractions 432 to 672.

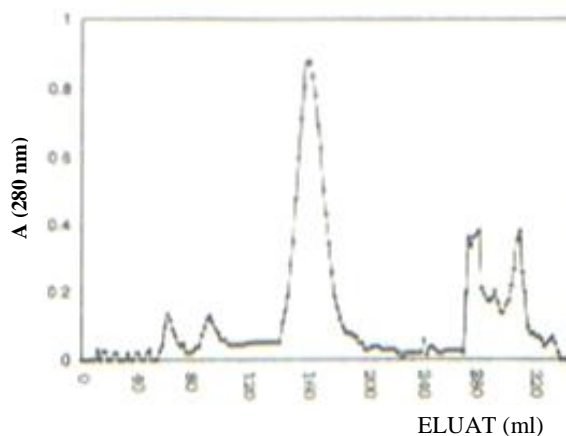


Fig. 2. Sephacryl S-200 column chromatogram of the concentrated PSA peak from CM-Sephadex chromatography. See the methods for the details of experimental procedures. Fractions 155-170 ml contained PSA.

Table 1. Comparison of PSA recovery and purity in different steps.

Steps	Sample volume (ml)	Total protein (mg)	Total PSA (mg)	Specific activity	Purity	% Recovery
Seminal plasma	20	735	10.86	0.0147	1	100
CM-Sephadex	1.5	71.2	9.4	0.132	8.97	86.5
Sephacryl S-200		6.8	6.8		68	62.5

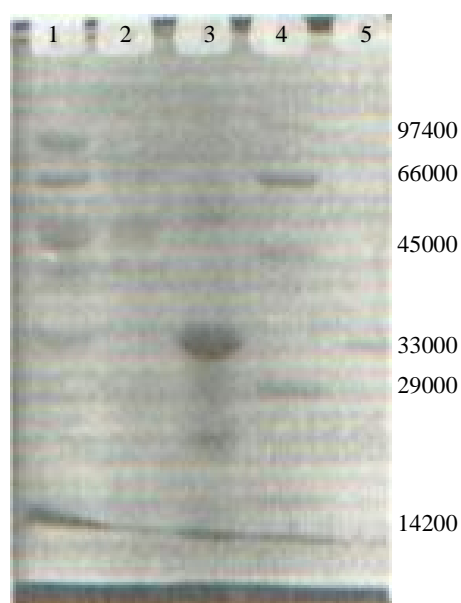


Fig. 3. SDS-PAGE of various fractions from different purification steps on a 10% polyacrylamide gel. See methods for details. Protein bands were visualized with Coomassie blue. Lane 1, seminal fluid; Lane 2, CM-Sephadex elute before establishing the gradient; Lane 3, CM-Sephadex elute after washing with linear salt gradient (between 432 ml and 672 ml fractions); Lane 4, molecular weight markers: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20 kDa); Lane 5, purified PSA from Sephacryl S-200 column.

Multiple protein bands were observed in the eluate of CM-Sephadex column (Figure 3, lane 3). However, upon SDS-PAGE analysis, only one protein band was observed from fractions 155 to 170 ml from Sephacryl S-200 column (Figure 3, lane 5). The purified PSA was shown to have a molecular weight of 33 kDa by SDS-PAGE. In the second method, the pooled fractions from Sephacryl S-200 column were subjected to HPLC and produced a major symmetrical protein peak with a higher retention times are probably related to fragmented pieces of the PSA.

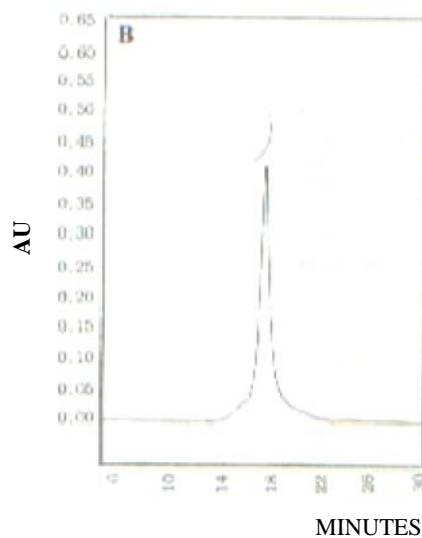
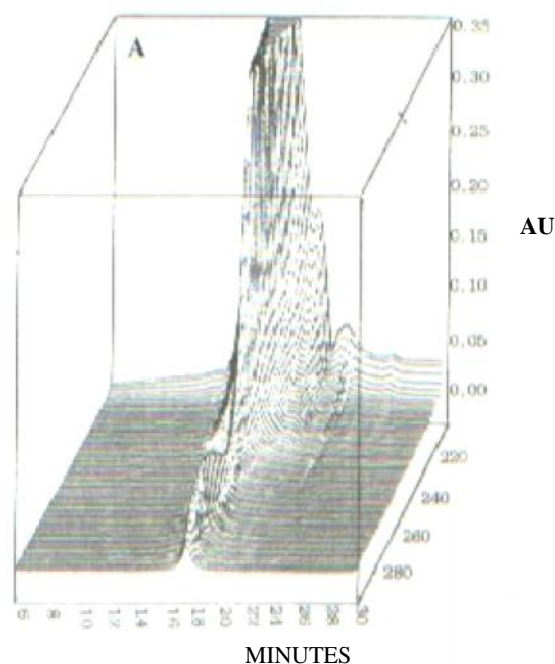


Fig. 4. (A) Three dimensional elution pattern of purified PSA from an HPLC column at 200-300 nm. For experimental details see Methods. The diagram shows the absorbance of the elutes between 200-300 nm (Au) vs. retention times. PSA was eluted in a major peak with a retention time of 17 min. The two shoulders with higher retention times detected at 200 nm are probably due to the absorbance of PSA fragments. (B) Two dimensional HPLC elution pattern of purified PSA at 280 nm. As seen, PSA was eluted in a major peak with a retention time of 17 min.



Fig. 5. Isoelectric focusing of Sephacryl S-200 purified PSA on a 4% polyacrylamide gel. The pH of the top and the bottom of the gel was 3.5 and 9.5, respectively. See methods for experimental procedure.

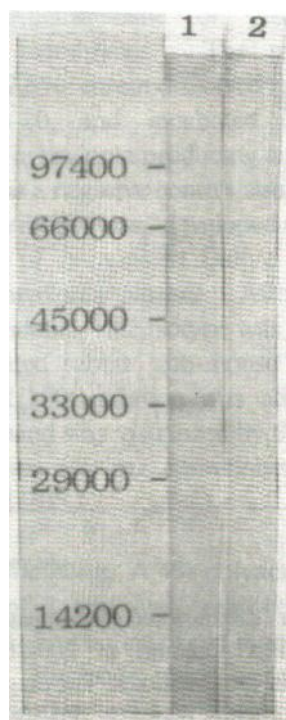


Fig. 6. Western blot of the purified PSA using anti-PSA monoclonal antibody. See methods for the detail of experimental procedure. Lane 1, the reaction of purified PSA with anti-PSA monoclonal antibody; Lane 2, reaction of purified PSA with anti-gp63 monoclonal antibody (a negative control).

time of 17 minutes (Figure 4A and 4B). The observed shoulders with higher retention times are probably related to fragmented pieces of the PSA.

Detection of PSA isoforms. As shown in Figure 5, the purified PSA from seminal plasma possessed at least five different isoelectric points. The major isoform is located toward the anode.

Immunoreactivity of purified PSA. For the determination of the immunoreactivity of the purified PSA, a specific monoclonal anti-PSA antibody (produced in our laboratory) was used. The fraction obtained from Sephacryl S-200 column was subjected to SDS-PAGE and after blotting of proteins onto the nitrocellulose membrane sections were incubated with anti-PSA monoclonal antibody observing a major band of 33 kDa (Figure 6, lane 1).

DISCUSSION

Various purification methods have been employed for the purification of PSA from prostate tissue and seminal plasma [9-12, 4]. In the current investigation a combination of ion-exchange chromatography and gel filtration were found to be an effective procedure for the purification of PSA with a recovery of 62.8%. This purified antigen is shown to be homogeneous by polyacrylamide gel electrophoresis and HPLC. It has a molecular weight of 33000 Dalton and exhibits at least five isomers.

This is the first report describing a simple method for the purification of all PSA isoforms. Wang and his associates [10] purified PSA using a five-step procedure. However, after isoelectric focusing, they detected only one PSA isoform with a pI of 6.9. In another experiment in order to simplify PSA purification, Wang *et al.* [13] used a four-step method with a recovery of 17%. They again observed a single isoform of this protein with an isoelectric point of 6.9. Recently, Chen *et al.* [14] using Sephacryl S-200 and CM-Sephadex C-50 have purified PSA from benign prostate hyperplasia (BPH) nodule fluid. They used Sephacryl S-200 gel filtration chromatography in the first step and removed the contaminating proteins in the PSA immunoreactive peak by CM-Sephadex column. They reported that the purified PSA from BPH nodule fluid had a single band on SDS-PAGE with a recovery of 64.6%. They did not evaluate the number of isoforms in this report. Zhang *et al.* [15] purified PSA from seminal plasma with a recovery of 30% and characterized the isoforms by anion-exchange

chromatography. During the third step of their purification using a Resource Q column they identified five isoforms of PSA. Comparing our method with that of Zhang *et al.* [15], it is revealed that our two-step method is simple, non-expensive and provides a high recovery (62.8%) of the purified PSA.

Our results reflect the molecular charge and size relationship between the PSA and the other proteins in human semen. The most acidic isoform of PSA has an isoelectric point of 6.8, while most of the other seminal proteins are more acidic. Therefore, in our first step of purification, all isoforms of PSA bind to CMSEphadex, whereas most of the other proteins were eluted in the void volume. With regard to size, human seminal plasma PSA is the major protein in the 20,000 to 40,000 molecular weight range as shown by SDS-PAGE analysis (Figure 3). Therefore, when the fractions containing PSA were further fractionated using the high resolution matrix Sephacryl S-200, a major symmetrical protein peak corresponding to PSA was obtained.

Most of the reported procedures for PSA purification are time-consuming and their recovery is only between 10 to 17%. The present purification method is rapid and provides a high yield of the homogenous PSA. From 20 ml of seminal plasma, 6.8 mg of PSA, corresponding to a recovery of 62.8% is obtained.

The reactivity of the purified PSA was checked by Western blotting. As a result, a 33 kDa band was observed (Figure 6). However, when PSA degraded at room temperature, at least two bands with lower intensities were also seen (data not shown). These latter bands are probably related to the fragmented parts of PSA that had retained their reactivity to anti-PSA monoclonal antibody. It is noteworthy to mention that the small shoulders observed in HPLC analysis may also be due to the presence of these minor bands.

ACKNOWLEDGEMENT

This work was supported by a grant number 76-438 from Shiraz University of Medical Sciences.

REFERENCES

- Lilja, H., Abrahamsson, P.A. and Lundwall, A. (1989) Seminogelin, the predominant protein in human seminal semen: primary structure and the identification of closely related proteins in the male accessory sex glands and on the spermatozoa. *J. Biol. Chem.* 264: 1894-1900.
- Armbruster, D.A. (1993) Prostate-specific antigen: biochemistry, analytical methods and clinical application. *Clin. Chem.* 37: 1618-1625.
- Kojima, M. and Babaian, R.J. (1995) Algorithms for early detection of prostate cancer. *Cancer* 75: 1860-1868.
- Lilja, H. (1985) A kallikrein-like serine protease in prostate fluid cleaves the predominant seminal vesicle protein. *J. Clin. Invest.* 76: 1899-1903.
- Cohen, P., Graves, H., Peehl, D.M., Kamarei, M., Giudice, L.C. and Rosenfeld, R.G. (1992) Prostate-specific antigen (PSA) is an insulin like growth factor binding protein-3 proteinase found in seminal plasma. *J. Clin. Endocrinol. Metab.* 75: 1046-1053.
- Webber, M.M., Waghray, A. and Bells, D. (1995) Prostate-specific antigen, a serine protease, facilitates human prostate cancer cell invasion. *Clin. Cancer Res.* 1: 1089-1094.
- Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with Folin phenol reagent. *J. Biol. Chem.* 193: 265-275.
- Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680.
- Schaller, J., Akiyama, K., Tsuda, R, Marti, T. and Rickli, E.E. (1987) Isolation, characterization and amino-acid sequence of gamma-seminoprotein, a glycoprotein from human seminal plasma. *Eur. J. Biochem.* 170: 111-130.
- Wang, M.C., Valenzuela, L.A., Murphy, G.P. and Chu, T.M. (1979) Purification of a human prostate-specific antigen. *Invest. Urol.* 17:159-163.
- Watt, K.W., Leep-J, M., Timkulu, T., Chen, W.P. and Loor, R (1986) Human prostate-specific antigen: structural and functional similarity with serine proteases. *Proc. Natl. Acad. Sci. USA.* 83: 3166-3170.
- Rusciano, D., Berardi, A., Ceccarini, C. and Terrana, B. (1988) Concomitant purification of prostatic carcinoma tumor markers from human seminal fluid under non-denaturing condition. *Clin. Chem.* 34:2528-2532.
- Wang, M.C., Valenzuela, L.A., Murphy, G.P. and Chu, T.M. (1982) A simplified purification procedure for human prostate antigen. *Oncology* 39:1-5.
- Chen, Z., Chen, H. and Stamey, T.A. (1997) Prostate specific antigen in benign prostatic hyperplasia: purification and characterization. *J. Urol.* 157.:2166-2170.
- Zhang, W.M., Leinonen, J., Kalkkinen, N., Dowell, B. and Stenman, U.H. (1995) Purification and characterization of different molecular forms of prostate-specific antigen in human seminal fluid. *Clin. Chem.* 41: 1567-1573.