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Short communication

Thiophilic interaction chromatography of prostate-specific antigen

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Abstract

Prostate-specific antigen (PSA) protein and complexes of PSA with α_1-antichymotrypsin (PSA-ACT) or α_2-macroglobulin (PSA-A_2M) prepared in vitro, have strong affinity for different thiophilic gels (T-gel). Free PSA, and these PSA complexes can be isolated due to their affinity for T-gels. The average recovery of PSA from several of the T-gels, based upon ELISA measurements, was 84 to 94%. The data suggest that T-gel affinity can be explored for the purification of free and complexed PSA from various biologic fluids.

Keywords: Thiophilic interaction chromatography; Prostate-specific antigen

1. Introduction

Prostate-specific antigen (PSA) is a kallikrein-like 30 kDa serum protease produced almost exclusively by the epithelium of the human prostate gland which is secreted into seminal fluid [1,2]. In recent years, PSA has become a unique serum tumor marker for the early detection and management of prostate cancer [3,4]. The majority of PSA in the serum is complexed with protease inhibitors such as α_1-antichymotrypsin (PSA-ACT), α_2-macroglobulin (PSA-A_2M) and α_1-antitrypsin (PSA-AT). However, the PSA-ACT complex is the only one detectable in significant amounts in the serum [5,6].

The currently available methods for the isolation of free-PSA and its complexes only result in yields of 3 to 20% of the total PSA present in the starting material [7–9]. Moreover, not all PSA complexes are recovered by any one method of isolation. In order to develop a simple and efficient method to isolate free-PSA and its molecular complexes present in biologic fluids, we have explored the thiophilic interaction chromatography (TIC) technique originally introduced by Porath and his colleagues [10].

Thiophilic gels (T-gel) are divinyl sulfone-activated agarose preparations treated with mercaptoethanol and have preferential affinity for immunoglobulins [11,12]. T-gels can be used for isolation of the protein with 'thiophilic regions' and peptides with 'aromatic amino acid residues' [13]. The chromatographic method is based upon salt-promoted adsorption of proteins, whereas desorption is achieved when the salt concentration is reduced [13]. The use of T-gel for affinity chromatography is not restricted to immunoglobulins; other proteins like α_2-macroglobulins, yeast-acetolactate, papain [14] and horse allergens [15] have been shown to have affinity for T-gels.

The affinity of PSA for a T-gel has not yet been

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reported. We find that free PSA and its complexes, PSA-ACT and PSA-A_2M, have strong affinity for all commercially available T-gels (PyS, 2S and 3S). The recovery of PSA is approximately 90% from all T-gels.

2. Experimental

2.1. Thiophilic gels and other chemicals

PyS-resin and a T-resin (2S) were purchased from Sigma Chemical Company, St. Louis, MO, USA. Fractogel-EMD TA (5) (3S) was purchased from EM Separation Science (Gibbstown, NJ, USA). Free-PSA was obtained from three different commercial sources: Sigma (St. Louis, MO, USA), Calbiochem (San Diego, CA, USA) and Chemicon (Temecula, CA, USA). α_1-antichymotrypsin and α_2-macroglobulin were from Sigma. All reagents for electrophoresis and Western-blot analysis were obtained from Bio-Rad Labs (Hercules, CA, USA). All other chemicals and reagents used were analytical grade, or otherwise of the highest purity available.

2.2. Antibodies

Mono- and polyclonal anti-PSA antibodies, polyclonal anti-α_1-antichymotrypsin antibody and polyclonal anti-α_2-macroglobulin antibody were purchased from Dako (Carpinteria, CA, USA). Peroxidase conjugated goat anti-mouse IgG and goat anti-rabbit IgG were purchased from Jackson Immuno Research Laboratories (West Grove, PA, USA).

2.3. Protein determination

Protein concentration was determined using the bicinchoninic acid (BCA) protein assay reagents (Pierce, Rockford, IL, USA) using 96-well microtiter plates [16]. Bovine serum albumin was used as a standard.

2.4. T-gel chromatography

The T-gel slurry (PyS, 2S or 3S T-gel) was packed in a BioRad column (0.5×5 cm) and equilibrated with 25 m*M* Hepes buffer containing 1 *M* sodium sulfate, pH 7.0 (column buffer). Ten micrograms of commercial PSA (Sigma, Calbiochem or Chemicon), reconstituted in the column buffer were applied to the column. The column was developed at a flow-rate of 10 ml/h. The column was washed with 20 ml of column buffer followed by 40 ml of a linear gradient of decreasing concentrations of sodium sulfate (l *M* to 0.0 *M*). In all cases, material was finally eluted from the column with 10 ml of 25 m*M* Hepes buffer (pH 7.0) containing no sodium sulfate. One milliliter fractions were collected.

2.5. Preparation of PSA complexes

PSA-ACT and PSA-A_2*M* complexes were made in vitro by mixing 10 μg of PSA (Sigma) with 20 μg of either α_1-antichymotrypsin or α_2-macroglobulin in phosphate buffered saline (PBS) containing 0.1% albumin at 37°C for 30 min. Each of the mixtures containing free and complexed PSA was then chromatographed on 3S T-gel.

2.6. SDS–PAGE and Western-blot analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) was performed by the method of Laemmli [17]. Proteins were electrophoresed under non-reducing conditions through 4–15% gradient-gel and transferred to polyvinylidene difluoride (PVDF) paper using established procedures [18]. The blots were probed with antibodies against PSA, α_1-antichymotrypsin or α_2-macroglobulin. Chemiluminescence reagent (New Life Science Products, Boston, MA, USA) was used to visualize the positive bands.

2.7. PSA measurement by ELISA

Determination of total PSA in various chromatographic fractions was carried out using specific sandwich enzyme-linked immunosorbant assay (ELISA). The assay uses a polyclonal anti-PSA antibody as the ‘capture’ antibody. The ‘detection’ antibody was monoclonal anti-PSA antibody. The assay was calibrated with purified PSA (Sigma); a good linear correlation was obtained in the range of 0.75 to 25 ng PSA/ml. Microtiter plates (Nunc-

Immuno plate, Maxisorp. Nunc Inc. Naperville, IL, USA) were coated overnight at 4°C with 50 μ1 of the appropriate dilution of capture antibody in PBS. 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 were added to duplicate wells and incubated for 1 h. For detection, 50 μl of the 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, a substrate solution containing 0.01% H_2O_2, and 0.4 μg/ml of *o*-phenyldiamine in 0.2 *M* citrate buffer, (pH 5.0), was added to each well. The reaction was stopped by adding 2N H_2SO_4, and the intensity of the color was measured at 490 n*M* in a plate reader.

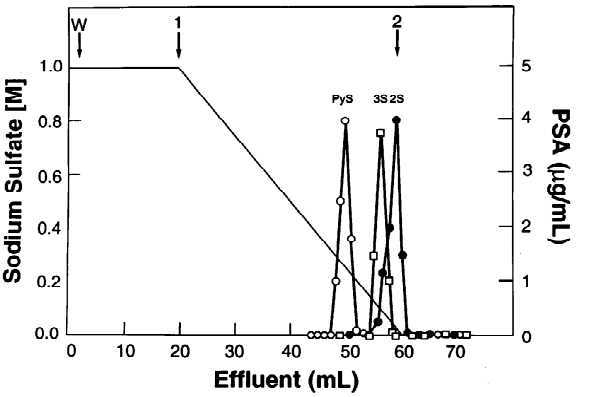

Fig. 1. Relative affinity of PSA (Sigma) for thiophilic gels. Ten micrograms of PSA was chromatographed on PyS, 2S or 3S T-gels. Each column was equilibrated with 25 m*M* Hepes buffer, containing 1 *M* sodium sulfate, (pH 7.0). PSA was retained on the column and eluted with a linear gradient of decreasing salt (1.0 to 0.0 *M* sodium sulfate). PSA concentration in various column fractions was determined by ELISA.

3. Results

3.1. Chromatography of PSA on thiophilic gels (T-gels)

Ten micrograms of commercially available PSA was chromatographed on 0.5×5 cm columns of PyS, 2S or 3S T-gels, as outlined above. The results are shown in Fig. 1. PSA was effectively retained on all three thiophilic gels and was eluted when the sodium sulfate concentration was reduced to 0.25 *M* for PyS, 0.05 *M* for 2S, and 0.1 *M* for 3S T-gel. Evidently, binding of PSA is weaker on PyS gel as compared to 2S and 3S T-gels. Purified PSA, obtained from commercial sources, seems to have somewhat greater affinity for 2S then 3S T-gel (Fig. 1). Based upon ELISA results, the average recovery of PSA from PyS, 2S and 3S T-gel columns was nearly 90% (Table 1). No differences in chromatographic behavior were seen on any of the T-gels when PSA from Calbiochem, Sigma, or Chemicon were chromatographed under identical conditions.

3.2. Affinity of PSA complexes for T-gel

PSA-ACT and PSA-A_2M complexes were made in vitro by mixing 10 μg of PSA with 20 μg of α_1-antichymotrypsin or α_2-macroglobulin in PBS containing 0.1% albumin at 37°C for 30 min. Each of the mixtures, containing free and complexed forms of PSA, was chromatographed on 3S T-gel column as outlined above. Free-PSA, PSA-ACT and PSA-A_2M complexes were retained on T-gel and were eluted with buffer containing no salt. The results of Western-blot analysis (Fig. 2) document the presence of free-PSA, PSA-ACT and PSA-A_2M complexes before (A) and after (B) T-gel chromatography. No PSA was detected in the column buffer fractions. The presence of PSA-ACT and PSA-A_2M complex was also confirmed by anti-ACT and anti-A_2M antibodies (data not shown).

Table 1
Recovery of PSA from T-gels[a]

T-gel	PSA (μg)		% Recovery[b]
	Applied	Recovered	
PyS	10	8.9±6.3	89
2S	10	8.4±6.9	84
3S	10	9.4±4.3	94

[a] Based upon ELISA determination; (b) Average of three experiments.

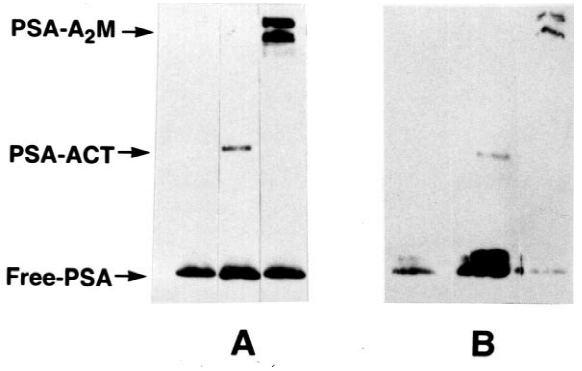

Fig. 2. Western-blot analysis of PSA-ACT and PSA-A_2M complexes (A) before and (B) after 3S T-gel chromatography. Ten micrograms of PSA (Sigma) was incubated at 37°C for 30 min with α_1-antichymotrypsin (20 μg) or with α_2-macroglobulin (20 μg) in PBS containing 0.1% albumin and chromatographed on 3S T-gel column. The column was equilibrated with a 25 m*M* Hepes buffer containing 1 *M* sodium sulfate, (pH 7.0). PSA and its complexes were eluted with a 25 m*M* Hepes buffer containing no sodium sulfate. After SDS–PAGE and Western transfer, the presence of free-PSA and PSA complexes was determined using the anti-PSA antibody.

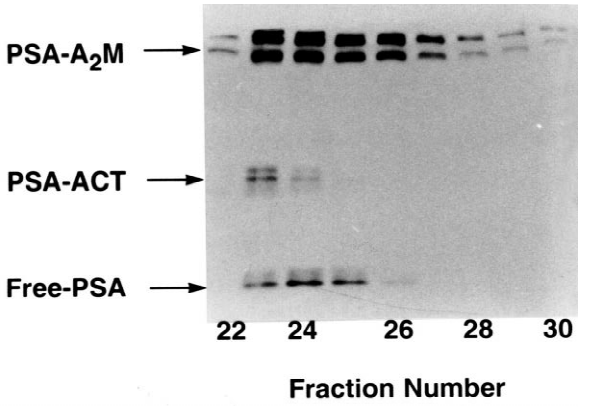

Fig. 3. Western-blot analysis of PSA molecular complexes present in the spiked female serum after 3S T-gel chromatography. Two hundred microliters of female serum was spiked with 5 μg of PSA (Sigma), and the mixture was incubated for 30 min at 37°C. This mixture was chromatographed on 3S T-gel as outlined for Fig. 2. No PSA immunoreactivity was seen in the wash region of the column: Three different molecular forms of PSA were identified in the elution region of the column, free PSA, PSA-ACT and PSA-A_2M. They were identified, by using anti-PSA, anti-ACT and anti-A_2M antibodies respectively. PSA-A_2M is the predominant complexed form in the spiked serum.

3.3. T-gel chromatography of female serum spiked with PSA

Thus far, we have shown that commercially available PSA and PSA-ACT or PSA-A_2M complexes prepared in vitro have strong affinity for T-gels. However, the T-gel affinity of PSA and its complexed forms when present in the serum may be different. Serum from a healthy female contains no detectable amount of PSA. Normal human serum, both male and female, is known to have abundant quantities of α_1-antichymotrypsin (0.5 μg/ml) and α_2-macroglobulin (3 μg/ml). PSA-ACT and PSA-A_2M complexes have been previously identified in spiked female serum [19,20].

In our studies, female serum (200 μl) was spiked with 5 μg of free-PSA, incubated for 1 h at 37°C and immediately chromatographed on 3S T-gel column as described in Fig. 2. The identity of PSA and its complexed forms was confirmed as described above. No PSA activity was detected in the column buffer region. PSA and its complexed forms were all retained on the column. The results are shown in Fig. 3. In the eluted fractions, free PSA and the two complexes, PSA-ACT and PSA-A_2M, were identified (Fig. 3). The T-gel affinity of free PSA and these complexed PSA forms in serum is similar to commercial PSA and complexes prepared in vitro. After 1 h of incubation, the majority of PSA added to the female serum was converted to a PSA-A_2M complex (Fig. 3). Two bands of immunoreactivity seen for free PSA and its complexed forms is due to the fact that PSA is known to have different isomeric forms primarily due to differences in glycosylation [26,27]. The identity of PSA-ACT and PSA-A_2M complexes was also confirmed independently with anti-ACT and anti-A_2M antibody in an ELISA test.

4. Discussion

Serum testing of PSA has become a powerful tool for screening and management of prostate cancer. Different commercial PSA immunoassays currently available, give different results in the same patients [21]. This underscores the need to standardize PSA assays. The first step towards standardization of PSA immunoassays is a standard method of purification of ‘native PSA’ and a method to preserve this native

form for a longer period of time. Also, a serum based 'reference material' containing a specified amount of PSA is currently in great demand [25].

Most of the currently available sources of PSA are from seminal plasma. PSA is purified by multiple-step procedures that involve techniques like ion-exchange chromatography, size exclusion chromatography, lectin affinity chromatography and chromatofocusing [7–9,22]. The overall recovery varies anywhere from 3 to 20% [7–9]. In our studies, we have shown that commercially available PSA has a strong affinity for the thiophilic gels: PyS, 2S and 3S with recoveries of 84–94% (Fig. 1 and Table 1). The PSA-ACT and PSA-A_2M complexes also have substantial affinity for T-gels (Figs. 2 and 3). Thiophilic interaction chromatography, therefore, offers an opportunity to isolate both free PSA and PSA complexes in a simple one-step procedure from biological fluids.

The affinity of a protein for T-gels is due to the presence of clusters of aromatic residues [10,13] on its surface. The primary structure of the PSA molecule reveals the presence of putative clusters of aromatic amino acids [23,24]. Whether or not these clusters of aromatic amino acid residues are accessible on its surface has to wait until the three dimensional structure of PSA is resolved.

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