Concomitant Purification of Prostatic Carcinoma Tumor Markers from Human Seminal Fluid under Nondenaturing Conditions

Dario Rucianu, Anna Berardi, Costante Ceccherini, and Benedetto Terrana

We report a protocol for concomitant purification to homogeneity of both prostatic acid phosphatase [orthophosphoric-monoester phosphohydrolase (acid optimum), EC 3.1.3.2] and prostate-specific antigen, from human seminal fluid. The method requires only two chromatographic steps: passage through an Affigel-Blue column and gel filtration HPLC. This is a fast, efficient procedure for purification of these two important tumor markers, which are specific for prostatic cancer.

Additional Keyphrases: acid phosphatase - prostate-specific antigen - cancer - chromatography, affinity gel

Carcinoma of the prostate gland is very common in elderly men—the third most common cause of death from cancer for men in the U.S. (1). Early diagnosis is advantageous, with both survival and therapeutic response being better in patients with early-stage disease than in those with metastatic cancer. Unfortunately, at the time of diagnosis 60% of patients present with advanced disease, 84% of them affected by metastatic dissemination (2).

Prostatic carcinoma was the first form of cancer to be associated with a tumor marker in serum, when it was discovered that malignant prostatic cells cause increases in the concentration of total acid phosphatases in the bone marrow of a patient with metastatic prostate cancer (3, 4). To date, many communications (e.g., 5–8) have emphasized the clinical relevance of high concentrations in serum of that peculiar acid phosphatase that originates in the prostate [orthophosphoric-monoester phosphohydrolase (acid optimum); EC 3.1.3.2; PAP].

More recently, a new tumor marker purified from human prostate tissue has been described (9): prostate-specific antigen (PSA), a glycoprotein peculiar to the epithelial cells of the prostatic duct and acini. PSA has a molecular mass of 33–34 kDa and an isoelectric point between pH 6.8 and 7.5 (10). It can also be isolated from seminal plasma, in a form reportedly identical to that in the prostate gland (10). The pretreatment PSA concentration is considered to be more reliable than PAP as a prognostic indicator of patient survival and disease progression (11, 12). However, Loor et al. (13) reported that the combined immunodetermination of both PAP and PSA in the serum appeared to increase the sensitivity and the specificity of the diagnostic test.

The methods described for purification of PAP (5, 14, 15) and PSA (9, 16) from seminal fluid and from prostate tissue are time consuming, and the final yield is often quite low. In particular, no recoveries of PSA have exceeded 17%.

We recently published a one-step method by which a high proportion of native PAP can be recovered from human seminal fluid (17). Here we describe a protocol for concomitant, high-yield purification of both PAP and PSA from human seminal fluid, under nondenaturing conditions.

Materials and Methods

Preparation of human seminal plasma: Pooled ejaculates from healthy donors were treated as described (17), except for the dialysis buffer, which contained 0.1 mmol of phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, MO) per liter, to inhibit the proteolytic activity of PSA (18).

Chromatography: Dialyzed seminal fluid (17) was loaded onto five volumes of Affigel-Blue gel (Bio-Rad Labs, Richmond, CA) packed in a column previously equilibrated with 50 mmol/L Na2HPO4 solution, buffered at pH 6.4 with 0.1 mol/L citric acid and containing phenylmethylsulfonyl fluoride, 0.1 mmol/L. The flow rate was 20 mL/h. We washed the column with the equilibrating buffer until no significant absorbance at 280 nm was detectable by a variable-wavelength spectrophotometer (LKB, Bromma, Sweden). We then washed the column with the equilibrating buffer containing NaCl, 0.75 mol/L, to detach mildly retained proteins. Finally, we eluted with equilibrating buffer containing 0.5 mol of KSCN per liter, to recover strongly retained proteins. Proteins eluting in the three peaks were separately pooled, and those effluents and eluates containing NaCl or KSCN were dialyzed for 24 h against three 5-L changes of the equilibrating buffer. Each of the three pools was then concentrated by using concentrators and "Diaflo" ultrafiltration membranes (molecular-mass cutoff, 10 kDa; Amicon Corp., Danvers, MA). Concentrated protein solutions (in a volume not exceeding 0.5 mL) were loaded onto a semipreparative gel-filtration HPLC column (BioSil TSK 250, 600 × 21.5 mm; Bio-Rad) under the described conditions (17), except that the flow rate was 3 mL/min, and the eluting buffer contained phenylmethylsulfonyl fluoride, 0.1 mmol/L. The column effluent was monitored at 280 nm, and 1.5-mL fractions were collected every 30 s with a fraction collector. The HPLC column had been previously calibrated under the same conditions by using the molecular-mass calibration kit from Bio-Rad.

For the analytical evaluation of purified PSA we used two analytical TSK 3000 SW columns (LKB), one 600 × 7.5 mm and the other 300 × 7.5 mm, connected in series (total length 90 cm) at a flow rate of 0.75 mL/min.

Biochemical quantification of PAP and PSA: The enzymatic activity of PAP was determined by using p-nitrophenyl phosphate (Calbiochem, San Diego, CA) as substrate, as described elsewhere (17). When necessary, samples were diluted in acetate buffer (0.1 mol/L, pH 5.4) containing glycerol (300 mL). For immunoenzymatic determination of PAP we used a PAP-EIA kit (Abbott Labs., North Chicago, IL) according to the manufacturer's instructions. Samples to be assayed were diluted in citrate/phosphate buffer (50 mmol/L, pH 6.4) containing 300 mL of glycerol per liter.

For immunoradiometric determination of PSA we used the Tandem-R-PSA kit (Hybritech, Liège, Belgium) according to the manufacturer's instructions, plotting specific bound counts/min on the y-axis and nanograms of protein on the x-axis. Samples to be tested were diluted in Tris-buffered saline (per liter, 150 mmol of NaCl, 20 mmol of Tris, 0.1 mol/L, pH 7.6).

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buffered at pH 7.4 with HCl) containing 30 g of bovine serum albumin (Calbiochem) per liter (TBS/BSA). We measured radioactivity in a Minaxi Auto Gamma 5000 gamma counter (Packard Instrument Co., Downers Grove, IL).

Other methods: Proteins were determined according to the method of Lowry et al. (19) with bovine serum albumin (Calbiochem) as standard.

Sodium dodecyl sulfate/polyacrylamide gel electrophoresis under reducing conditions (20) was performed on 100 g/L slab gels, with a Bio-Rad mini-gel apparatus. Protein bands were stained with Coomassie Blue R-250 (Bio-Rad). For polyacrylamide gel electrophoresis under nondenaturing, nonreducing conditions we used a 50 g/L slab mini-gel at pH 9.5 (21). Proteins were then either stained with Coomassie Blue R-250, or blotted onto a nitrocellulose filter according to Towbin et al. (22), by applying 300 mA for 2 h with an LKB Macrodrive 1 power supply, Model 2301. The filter was saturated in TBS/BSA for 2 h at 37 °C, then incubated under the same conditions with 125I-labeled monoclonal antibody to PSA (contained in the Tandem-R-PSA kit from Hybritech), diluted in TBS/BSA to 500,000 counts/min. After washing the filter several times with TBS and BSA containing 1 mL of Tween 20 per liter, we exposed it to x-ray film (X-OMAT AR; Eastman Kodak Co., Rochester, NY) at −70 °C for 5 h, then developed the film in an X-OMAT M20 processor (Kodak).

All line drawings were performed with a MacIntosh II Computer (Apple Computer, Cupertino, CA) equipped with a Laser Writer Plus (Apple Computer).

Results

During preliminary experiments (not shown) we established that the Affigel-Blue matrix had the ability to retain PSA strongly; it could be detached only at a high concentration of a chaotropic ion, while most of the PAP emerged in the flow-through. This strong interaction between PSA and the dye allowed many contaminating proteins to be removed before elution of PSA. Figure 1 shows the elution profile of a dialyzed seminal fluid sample loaded onto the Affigel-Blue column. The first peak (peak A), representing unretracted proteins, contained 83.5% of the original PAP, and only 0.2% of the original PSA (Table 1). The second peak (peak B), in which mildly retained proteins are eluted by 0.75 mol/L NaCl, contained no detectable PAP enzymatic activity, and only 0.4% of the original PSA (Table 1). In the third peak (peak C), representing strongly retained material eluted by 0.5 mol/L KSCN, no PAP was detectable, but 73% of the original PSA could be accounted for (Table 1).

Proteins eluted in the three peaks were concentrated separately to smaller volumes (see Materials and Methods and Table 1), and then injected in 0.5-mL aliquots onto the HPLC gel filtration column. Figure 2 shows the HPLC elution profile of the flow-through material (peak A); PAP emerged as a single, well-separated peak at around 42 min, as revealed by the evaluation of the enzymatic activity in the various fractions (Figure 3, dotted line). Fractions collected between 41 and 44 min were combined as pure PAP. The resolution of PAP from the peak containing serum albumin (around 47 min, Figure 3) is improved, as evidenced by the comparison of the above elution profile and that obtained by direct injection of seminal fluid onto the HPLC gel filtration column (17), because it is well known that the Blue A matrix can bind serum albumin (23), whereas PAP, at least under the conditions described, is not retained. Figure 3 shows the HPLC elution profile of peak C. PSA was eluted as a single peak around 56 min, as shown by the radioimmunodetection (see Materials and Methods) of the various fractions (Figure 3, dotted line). Fractions eluting between 54 and 59 min were pooled, concentrated, and an aliquot was re-injected onto two HPLC analytical gel-filtration columns connected together (total length 90 cm). PSA again was eluted as a single, symmetrical peak, thus indicating the purity of the loaded material.

The purity of both PAP and PSA recovered after HPLC chromatography was further assessed on a 100 g/L sodium dodecyl sulfate/polyacrylamide slab mini-gel run under reducing conditions. Figure 4 shows the electrophoretic profile of proteins during the various purification steps. The electrophoretic pattern of dialyzed seminal plasma total proteins are shown in lane B, whereas lanes C, D, and E show respectively the proteins eluted in peaks A, B, and C of the Affigel-Blue column. Clearly, the unretracted material is greatly enriched in PAP, and peak-C material contains a predominant band with a molecular mass of 34 kDa, whereas peak-B material (that emerging on washing the column with 0.75 mol/L NaCl) shows essentially no PAP or PSA. Lanes F, G, and H, respectively, show PAP and two different preparations of PSA, purified after passage through the HPLC gel-filtration column.

Figure 5 shows the purified PSA electrophoresed on a 50 g/L polyacrylamide gel under non-denaturing, nonreducing conditions. In lane A the protein pattern is revealed by Coomassie Blue staining, whereas lane B shows the result of the autoradiography after specific radioimmunostaining of the protein blot. Four corresponding major bands are visible by each of the two methods, suggesting isomeric protein heterogeneity but further confirming the purity of the isolated PSA, because each protein band is also revealed by the monoclonal antibody staining.

We do not show further evidence of the purity of the PAP thus obtained, considering that in the present schedule the material we loaded onto the HPLC gel filtration column for PAP purification contained less contaminating protein than did the crude material we loaded onto the same HPLC column in our previous work (17) where PAP purified in one step was judged to be pure by different criteria.

Under the conditions described, the entire purification of PAP from peak-A material required seven injections onto the BioSil TSK semipreparative column, yielding about 4 mg of pure enzyme. The entire purification of PSA from
Table 1. Purification Steps in the Preparation of Homogeneous Prostatic Acid Phosphatase and Prostate Specific Antigen from Human Seminal Fluid

<table>
<thead>
<tr>
<th></th>
<th>Seminal fluid</th>
<th>Affigel-Blue</th>
<th>HPLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vol, mL</td>
<td>4.5</td>
<td>3.6*</td>
<td>4.0*</td>
</tr>
<tr>
<td>Total protein, mg</td>
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<td>1.35</td>
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<td>Mass, mg</td>
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<tr>
<td>Activity, U</td>
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<td>1985</td>
<td>n.d.</td>
</tr>
<tr>
<td>Spec. acty, U/mg protein</td>
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<td>79.4</td>
<td>n.d.</td>
</tr>
<tr>
<td>Purifn. factor</td>
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<td>n.d.</td>
</tr>
<tr>
<td>Mass, mg</td>
<td>3.0</td>
<td>0.006</td>
<td>0.012</td>
</tr>
<tr>
<td>Purifn. factor</td>
<td>1</td>
<td>12.5</td>
<td>2.2</td>
</tr>
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* After concentration (see Materials and Methods).

PAP

1. Determined with the Lowry method (18).
2. Determined with an immunoenzymatic kit (Abbott).
3. Determined with an immunoradiometric kit (Hybtech).
4. n.d. = not done.

peak-C material required three injections, yielding about 1.4 mg of pure protein.

After concentration of the PAP-containing fractions to about 4 mL, we could account for 62% of the immunological reactivity (as measured by the Abbott immunoenzymatic assay), and 58% of the enzymatic activity (Table 1). After concentration of PSA-containing fractions to about 1.4 mL, the analytical recovery of purified protein was 45%, as revealed by the immunoradiometric determination (Table 1).

Discussion

The present method represents a substantial improvement in the purification of specific tumor markers for prostatic carcinoma.

Our earlier one-step protocol for the purification of PAP from human seminal fluid by HPLC gel filtration (17) resulted in a very fast recovery of about 70% of the enzyme. Under these conditions (17) we resolved PAP from other seminal-fluid proteins, although there was some overlapping with the serum albumin peak. On the other hand, the PSA activity was contained in a broad protein peak eluting between 20 and 28 min (17, data not shown). Because our preliminary data showed that an Affigel-Blue column could retain with different strength both serum albumin and PSA, while PAP was not notably retained, we decided to exploit this particular property of the matrix in trying to purify the two tumor markers from the same seminal fluid sample.

The exact mechanism by which proteins are resolved by chromatography on immobilized Blue A is still unclear. Owing to the characteristics of the sulfonated dye, Cibachron Blue F3-GA, covalently coupled to cross-linked agarose gel, this matrix is expected to interact with proteins according to one of the following mechanisms, or some combination of them: (a) ion exchange, (b) hydrophobic interactions, (c) exclusion/diffusion, and (d) affinity binding (24, 25). About 35% and 30% of PAP and PSA, respectively, are made up of hydrophobic amino acids, and both of them are sialylated glycoproteins, although PAP seems to contain more sialic (neuraminic) acid residues per molecule than does PSA (5, 26). If ionic exchange and hydrophobic binding were the cause of interactions with the dye, one would expect a stronger retention of PAP than PSA; experimental results indicate the opposite (Figure 1). Thus, other mechanisms such as affinity binding should be evoked to explain the non-retention of PAP and the tenacious interaction of PSA.

The purification of PAP and PSA under the conditions described requires only three to four days, with a final yield of about 60% of PAP and 45% of PSA (Table 1) from the

![Fig. 2. Elution profile of 0.5 mL of peak-A material from Affigel-Blue column applied to the BioSil TSK 250 HPLC gel-filtration column](image)

Proteins in the effluent were monitored by their absorption at 280 nm (——) and by measuring enzymatic activity (-----). Molecular-mass standards were eluted as follows: thyroglobulin (669 kDa) 30 min, IgG (158 kDa) 41 min, ovalbumin (43 kDa) 50.5 min, myoglobin (17.2 kDa) 59.2 min, and cyanocobalamin (1.35 kDa) 71 min

![Fig. 3. Elution profile of 0.5 mL of peak-C material from the BioSil TSK 250 HPLC gel-filtration column](image)

Proteins in the effluent were monitored by their absorption at 280 nm (——) and by the PSA-specific immunoradiometric test (-----). Calibration of the column as in Fig. 1. Inset: Elution profile of 10 μg of purified PSA (fractions 54–59 of the above column) injected in two analytical TSK 3000 SW columns connected together, with effluent monitored at 280 nm.

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same sample of seminal fluid. The physical recovery of PAP was a little lower than that described in our previous paper (17), and should be interested only in purification of this enzyme, the one-step methodology (17) could be preferred, because it is faster and physical recovery is better. Nevertheless, the two-step method presented here also allows good recovery of the prostatic enzyme in a reasonably short time, with a yield that compares well with other reported methods (5, 15). Furthermore, it allows the concomitant purification of PSA. Procedures described hitherto for purifying this protein from seminal fluid or from the prostate gland required at best a nine-day purification schedule, with a final yield no greater than 17%. Thus, our proposed protocol, which allows 45% physical recovery of pure PSA after only three days, is a great improvement.

PSA purified according to the procedure described above migrated as a single band on sodium dodecyl sulfate/polyacrylamide gel electrophoresis under reducing conditions, with an apparent molecular mass of about 34 kDa (Figure 4), as expected (9). It is well known that folding of the molecule is important in determining protein migration on the gel; in the case of PSA, migration under nonreducing conditions results in an apparent molecular mass shift below 30 kDa (data not shown). The heterogeneity seen under nondenaturing, nonreducing conditions (Figure 5) could be explained by the reported discovery that there are different isomers of the protein, with pIs ranging from 8.8 to 7.5 (27). A variable sialic acid content seems to be the cause of this heterogeneity (27).

The relevance, reliability, and limitations of PAP as a serum marker for prostatic carcinoma have been widely described over the years (5, 11). PSA is a relatively new serum marker for this type of carcinoma, and its correlation with prostatic disease appears to be better than that of PAP (11, 12). However, not all prostatic tumors produce both PAP and PSA; most seem to produce only one (28), so the

Fig. 4. Sodium dodecyl sulfate/polyacrylamide electrophoresis of processed seminal fluid
Lane A: molecular mass standards (Pharmacia), top to bottom: phosphorylase B (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), and trypsin inhibitor (20 kDa). Lane B: dianalyzed seminal fluid (58 μg). Lane C: peak-A (see Fig. 1) material (51 μg). Lane D: peak-B (see Fig. 1) material (20 μg). Lane E: peak-C (see Fig. 1) material (17 μg). Lane F: purified PSA (fractions 41–44, 4 μg). Lanes G, H: purified PSA from two different HPLC pools (fractions 54–59, 3.6 and 2.7 μg, respectively).

Fig. 5. Polyacrylamide gel electrophoresis of purified PSA (5 μg) under nondenaturing, nonreducing conditions
Lane A, proteins stained with Coomassie Blue; lane B, PSA bands revealed by autoradiography after labeling with specific 125I-labeled monoclonal antibody

immunodetection in serum or in histological samples of both PAP and PSA should increase the reliability of the test (28). Thus, the availability of a rapid, efficient method for concomitantly purifying these two specific tumor markers from human seminal fluid should be helpful for the production and the standardization of the relevant diagnostic kit, and for a better characterization of the biological and physiological properties of these molecules.

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Calculation of Low-Density Lipoprotein Cholesterol with Use of Triglyceride/Cholesterol Ratios in Lipoproteins Compared with Other Calculation Methods

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Low-density lipoprotein cholesterol was calculated with a formula that utilizes the triglyceride/cholesterol ratios in the different lipoprotein fractions, and also with different modifications of the Friedewald formula. Results of the former calculation correlated well with the ultracentrifugation-derived values and performed better than the other calculations at different lipid concentrations.

Additional Keyphrases: Friedewald formula · values by ultracentrifugation compared

The Friedewald factor (FF) has been used extensively in deriving the calculated value of LDL-cholesterol (LDL-C) since the first publication of this use by Friedewald et al. (1). This very convenient method of estimating lipoproteins requires measurement of only three variables in serum or plasma: total cholesterol (TC), total triglycerides (TG), and HDL-cholesterol (HDL-C) after the apo-B associated lipoproteins, LDL and VLDL, have been precipitated. The calculation uses the ratio of VLDL-C to TG—i.e., the FF—to derive the LDL-C from TG. Thus the calculation assumes that there is a fixed relation between VLDL-C and TG, which can be true only if (a) the triglyceride/cholesterol ratio in the VLDL is constant and (b) only VLDL contributes to the TG pool. However, the triglyceride/cholesterol ratio in VLDL is known to vary widely (2) and the other lipoproteins and lipoprotein remnants of course contribute to the TG pool. It has been suggested that the FF be modified such that an intercept term is added (3), and the FF has been revised on the basis of a large set of observations (4, 5). But no attempt has been made to account for the contribution to TG by the non-VLDL lipoproteins in the calculation of LDL-C. Here we compare such a calculation and calculations made using modifications of the FF with results for LDL-C as determined by ultracentrifugation.

Methods

We used 196 sera submitted to the laboratory for "lipid profile." The lipoproteins were fractionated by sequential ultracentrifugation, and the fractions were aspirated with a fine Pasteur pipette (6). We then determined cholesterol and triglyceride in all the fractions as well as in the whole sera by enzymatic methods, using kits (BioMerieux, Charbonnieres les Bains, France). In the triglyceride method a molecular mass of 877 Da for triglycerides is assumed. We subtracted 0.11 mmol/L from all serum TG values as correction for free glycerol, as recommended by the manufacturer. The methods were linear in the range of concentra-

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2 Health Research Department, Ministry of Public Health, Kuwait.
3 Nonstandard abbreviations: FF, Friedewald factor; TC, serum cholesterol; TG, serum triglycerides; VLDL, LDL, HDL, very low-, low-, and high-density lipoproteins; C, cholesterol; and apo, apolipoprotein.

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