Purification and Characterization of Different Molecular Forms of Prostate-Specific Antigen in Human Seminal Fluid

Wan-Ming Zhang, Jari Leinonen, Nisse Kalkkinen, Barry Dowell, and Ulf-Håkan Stenman

We have developed a new procedure for purifying prostate-specific antigen (PSA) from human seminal fluid. The method is based on ammonium sulfate precipitation, hydrophobic interaction chromatography, gel filtration, and anion-exchange chromatography. It can be completed within 2 days with a recovery of intact PSA of 30%. By anion-exchange chromatography, five isoforms of PSA (A, B, C, D, and E) can be separated. The major form (PSA-B) consists of the intact enzyme, as shown by the occurrence of only one band of 33 kDa in sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing or nonreducing conditions, and by amino acid sequencing, which reveals only one amino-terminal sequence corresponding to the reported amino-terminal sequence of intact PSA. The specific absorbance of 1 g/L PSA-B at 280 nm was 1.61, and 80% of the PSA-B formed a complex with α1-antichymotrypsin, indicating that it is enzymatically active. Three cleaved forms of PSA with different nicking sites and low enzymatic activity were separated from intact PSA by ion-exchange chromatography. In addition, we isolated a glycosylation variant, PSA-A, which showed a higher isoelectric point (pl = 7.2) than PSA-B (pl = 6.9) but similar enzymatic activity; this form accounts for 5–10% of total PSA. After treatment with sialidase, PSA-A and B had the same isoelectric point value (pl = 7.7).

Indexing Terms: isoenzymes/enzyme activity/chromatography, reversed-phase/chromatography, hydrophobic interaction

Prostate-specific antigen (PSA) is a glycoprotein of ~30 kDa found mainly in prostatic tissue and seminal fluid (1–3). Recently, PSA immunoreactivity has also been detected in human paraurethral (4, 5) and anal glands (6) and even in breast tumor cytosol (7). PSA isolated from human prostatic tissue and its secretions consists of a single polypeptide backbone of 237 amino acids containing 5 disulfide bonds and ~8% carbohydrate composed of an N-linked oligosaccharide chain (1, 8, 9). Studies of enzyme activity have demonstrated that PSA is a chymotrypsin-like serine protease. A member of the glandular kallikrein family (1), it is able to form complexes with α1-antichymotrypsin (ACT) and α2-macroglobulin (10). The physiological function of PSA is to dissolve the seminal coagulum formed after ejaculation (11, 12).

PSA purified from seminal fluid is heterogeneous (9, 10, 13). About 65% of PSA in seminal fluid appears to be intact and have enzymatic activity, whereas 35% is inactive, apparently because of an internal cleavage of the peptide chain. This nicked form, therefore, does not react with protease inhibitors such as ACT (10).

We have shown previously that PSA in serum occurs both in a free form and as a complex with ACT (14). The proportion of the complex between PSA and ACT (PSA-ACT) is greater in serum from patients with prostatic cancer than in serum from those with benign prostatic hyperplasia (14–16). The presence of different molecular forms of PSA in serum complicates the standardization of PSA immunoassays, because various assay methods recognize free PSA and PSA-ACT differently (17). To develop methods measuring the different forms of PSA in serum equally, it is important to have pure standards of the various forms.

Preliminary studies suggested that various forms of free PSA in seminal fluid may react differently with different monoclonal antibodies (18). In this study, we have developed a rapid, high-yield purification procedure for PSA from seminal fluid and have characterized isofoms of PSA separated by anion-exchange chromatography.

Materials and Methods

Samples

Human semen was allowed to liquefy, after which the sperm were removed by low-speed centrifugation (600g, 10 min, 25 °C). The resulting seminal fluid was further clarified by high-speed centrifugation (35 000g, 20 min, 4 °C) and stored frozen (−20 °C) until used. Pooled female serum was prepared by mixing samples from 40 women. A serum with a high concentration of PSA was obtained from a patient with advanced prostatic cancer. All sera were aliquoted and kept frozen (−20 °C) until used.

Reagents

Phenyl-Sepharose-HP, Sephacryl-200, Superdex-200 (1.6 × 60 cm), and Resource Q columns (6 mL) were purchased from Pharmacia Biotech (Uppsala, Sweden). An 0.8 × 10 cm µBondapak C18 Radial PAK column was obtained from Waters Associates (Milford, MA).
Chromogenic substrates, N-benzoyl-L-isoleucyl-glutamyl-glycyl-L-arginine-p-nitroanilide (S-2222) and 3-carbomethoxypropionyl-L-arginyl-L-prolyl-L-tyrosine-p-nitroanilide (S-2556), were from Kabi (Stockholm, Sweden). Eu³⁺ chelate for labeling antibodies was supplied by Wallac (Turku, Finland). Polyvinyl difluoride membranes (Immobilon P) were from Millipore (Bedford, MA). Bovine trypsin, bovine chymotrypsin, and neuraminidase (type V, from Clostridium perfringens) were from Sigma (St. Louis, MO). The protein molecular mass and isoelectric point (pl) markers were from Pharmacia.

Preparation of ACT. ACT was partially purified from 2 mL of female serum by gel filtration on Superdex-200. Fractions containing ACT were pooled and concentrated by ultrafiltration with a centrifugal concentrator (Amicon, Beverly, MA).

Antibodies. Polyclonal antibodies to PSA and ACT and peroxidase-conjugated swine anti-rabbit IgG antibody were from Dakopatts (Copenhagen, Denmark). Two monoclonal antibodies (MAb) to PSA (H117 and H50) used to develop an assay for total PSA were produced at Abbott Laboratories. Cross-reactions of polyclonal antibodies were tested by immunodiffusion as described (14). The polyclonal antibody to ACT and the MAb (H50) to PSA were labeled with Eu³⁺ chelate as described (14).

Procedures

Immunassays. For determination of PSA in samples and chromatographic fractions, we developed a specific time-resolved immunofluorometric assay (IFMA) as described (14). Briefly, MAb H117 was adsorbed to the microtitration wells, and MAb H50 labeled with europium was used as a tracer. This assay measures free PSA and PSA-ACT in an equimolar fashion. Purified PSA (PSA-B) was used for calibration of this assay, the PSA content of this preparation having been determined by gravimetry (see below). The PSA-ACT assay also included H117 as solid-phase antibody but a polyclonal antibody to ACT labeled with europium was the tracer. The PSA-ACT concentration in a serum containing a high concentration of PSA was determined by IFMA for total PSA after separation of PSA-ACT and free PSA by gel filtration. Dilutions of this serum in a female serum pool were used to prepare calibrators for determination of PSA-ACT (16).

Electrophoretic methods. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed under reducing or nonreducing conditions (19) on thin, homogeneous 20% polyacrylamide gels with the PhastSystem (Pharmacia). Proteins were stained with silver. For determination of amino-terminal sequences of PSA fragments, SDS-PAGE was carried out on 8 × 8 cm 3–16% gradient polyacrylamide gels, 2 mm thick, followed by electrophoretic transfer of the proteins to Immobilon P. The proteins were visualized by staining with Coomassie Blue, and the bands were cut out and subjected to sequencing in a gas-pulsed liquid sequencer (20). Isoelectric focusing (IEF) was carried out with the PhastSystem on gels with a pH range of 3.0–9.0. Proteins were stained with Coomassie Blue. Purified samples of PSA were subjected to IEF before and after treatment with neuraminidase as described (21). Immunoblotting was performed essentially as described (22). Polyclonal PSA antiserum was used to probe PSA immunoreactivity after blotting of the gel to Immobilon P.

Measurement of enzyme activity. To measure the enzyme activity of PSA, we added 50 µg of PSA to 800 µL of 50 mmol/L Tris-HCl buffer, pH 8.0, containing 10 mmol/L calcium chloride and 1 mL Tween-100. The reaction was initiated by adding 200 µL of the same buffer containing 0.5 mmol/L chromogenic substrates. The absorbance was monitored at 405 nm for 30 min with a nine-channel FP-901 photometer (Labsystems, Helsinki, Finland).

Purification. Saturated ammonium sulfate solution (5 mL) was added to 15 mL of clarified seminal fluid (i.e., yielding 25% saturation), mixed for 30 min at 4 °C, and centrifuged (35 000g, 20 min, 4 °C). To the resulting supernatant we added 30 mL of saturated ammonium sulfate solution (i.e., now 70% saturation) and mixed for 30 min at 4 °C. The precipitate formed was collected by centrifugation (5000g, 20 min, 4 °C) and was dissolved in 60 mL of 50 mmol/L Tris-HCl, pH 8.0, containing 3 mmol/L sodium azide, 2 mmol/L benzamidine, and 0.8 mol/L ammonium sulfate (buffer A). The resulting solution was clarified by centrifugation (35 000g, 20 min, 4 °C) and was applied to a 10-mL Phenyln-Sepharose-HP column equilibrated with buffer A. The column was washed with three bed volumes of buffer A and eluted with a linear gradient of 30 mL of buffer A and 30 mL of buffer B (50 mmol/L Tris-HCl, pH 8.0, containing 3 mmol/L sodium azide and 2 mmol/L benzamidine and, 400 mL/L isopropanol). The flow rate was 1 mL/min, and 2-mL fractions were collected. Fractions containing PSA immunoreactivity were pooled and precipitated by addition of solid ammonium sulfate to 75% saturation. The precipitate collected by centrifugation (35 000g, 20 min, 4 °C) was dissolved in 10 mL of 10 mmol/L Tris-HCl, pH 8.0, containing 3 mmol/L sodium azide and 2 mmol/L benzamidine (buffer C), and applied to a Sephacryl S-200 column (2.6 × 85 cm) equilibrated with buffer C. The flow rate was 20 mL/h, and 5-mL fractions were collected. Fractions containing PSA immunoreactivity were pooled and then applied to a Resource Q column equilibrated with buffer C. The column was washed with five bed volumes of the same buffer. Bound proteins were eluted with a linear gradient of 60 mL of buffer C and 60 mL of buffer D (buffer C plus 0.3 mol/L NaCl). The flow rate was 2 mL/min, and 1-mL fractions were collected. PSA in the fractions was dialyzed against 10 mmol/L Tris-HCl, pH 8.0, containing 0.2 g/L sodium azide before measuring the enzyme activity.

Reversed-phase chromatography. Reversed-phase chromatography was performed essentially as described (9), but we used a C₁₈ instead of a C₂ column. The column was equilibrated with 1 mL/L trifluoroacetic acid and eluted with a 0–50% acetonitrile gradient.
Table 1. Recovery of PSA after each step of purification.

<table>
<thead>
<tr>
<th>Total protein</th>
<th>PSA, mg*</th>
<th>Purity, %*</th>
<th>Purification, -fold</th>
<th>Recovery, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seeded fluid</td>
<td>2720.0</td>
<td>33.9</td>
<td>2.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Centrifugation</td>
<td>2070.0</td>
<td>33.6</td>
<td>2.6</td>
<td>1.3</td>
</tr>
<tr>
<td>Precipitation</td>
<td>873.0</td>
<td>30.8</td>
<td>5.7</td>
<td>2.9</td>
</tr>
<tr>
<td>Hydrophobic interaction chromatography</td>
<td>107.0</td>
<td>24.8</td>
<td>37.3</td>
<td>18.7</td>
</tr>
<tr>
<td>Gel filtration</td>
<td>30.3</td>
<td>17.6</td>
<td>93.5</td>
<td>46.8</td>
</tr>
<tr>
<td>Anion-exchange chromatography</td>
<td>16.5</td>
<td>10.2</td>
<td>99.5</td>
<td>49.8</td>
</tr>
</tbody>
</table>

* The content of PSA at each step was determined by IFMA.
* After correction for specific absorbance of PSA at 280 nm (1.61 vs 1.0 for total protein).
* The recovery after the last step represents only the main component, PSA-B.

We injected into the column 5 nmol of PSA dissolved in 1 mL of starting buffer and then monitored the absorbance of the fractions at 280 nm and measured the PSA immunoreactivity in 1-mL fractions.

Amino-terminal sequencing. To determine the amino-terminal amino acid sequence, we further separated by SDS-PAGE the different forms of PSA obtained by anion-exchange chromatography. Protein bands transferred to the Immobilon P membrane were cut out and analyzed with a gas-pulsed liquid sequencer (20).

Determination of specific absorbance. Purified PSA was dialyzed against distilled water and aliquoted. The absorbance at 280 nm was determined from aliquots containing PSA at 0.1–0.5 g/L by using a Perkin-Elmer (Norwalk, CT) Lambda 3B spectrophotometer. Other aliquots were lyophilized thoroughly and weighed on a microbalance (AE-163; Mettler Instrumente, Zürich, Switzerland) before and after further drying under reduced pressure over silica gel.

Results

Purification of PSA

Precipitation with ammonium sulfate resulted in 2.9-fold purification (Table 1). When the precipitate was reconstituted and applied to Phenyl-Sepharose, most of the PSA (>99%) was retained, whereas most of the other proteins were in the flowthrough fraction (Fig. 1). Bound PSA was eluted when 2-isopropanol in the eluent was 150–200 mL/L. When the fractions containing PSA were further fractionated by gel filtration, a major symmetrical protein peak corresponding to the PSA immunoreactivity was obtained (Fig. 2). In SDS-PAGE, fractions 54–65 displayed a single band of ~33 kDa under nonreducing conditions (Fig. 3, left) and several bands under reducing conditions (Fig. 3, right). When the PSA peak from gel filtration was further fractionated by anion-exchange chromatography on a Resource Q column, five partially separated PSA peaks, designated PSA-A to PSA-E, were obtained (Fig. 4). The major peak (PSA-B) represented ~60% of the PSA immunoreactivity. Moreover, it represented ~30% and the other components (PSA-A, C, D, and E) together ~22% of the PSA (Table 1) in the starting material. Thus, total recovery of PSA was 52%. The absorbance at 280 nm of PSA-B was determined from an aliquot that was thoroughly dried and weighed. Extended drying in a desiccator with silica gel had only a minor effect (~1%) on the weight of PSA. The specific absorbance of PSA was 1.61 for a protein concentration of 1 g/L.

Characterization of the Different Forms of PSA

PSA-A and B (Fig. 3) showed a single 33-kDa band under reducing and nonreducing conditions on SDS-PAGE (lanes A-B in Fig. 3, both panels). In IEF, the pl of PSA-A was 7.2, and that of PSA-B was 6.9 (Fig. 5, left). Neuraminidase treatment increased the pl of PSA-A and PSA-B to 7.7 (Fig. 5, right). Amino acid sequencing showed that PSA-A and PSA-B both had a single, identical amino-terminal sequence, Ile-Val-Gly-Gly- (Table 2), which corresponds to the reported amino-terminal sequence of intact PSA (1, 23). PSA-A and PSA-B were homogeneous in reversed-phase chromatography, as evidenced by a single

![Figure 1. Hydrophobic interaction chromatography of PSA precipitated from seminal fluid with ammonium sulfate.](image-url)
protein peak that corresponded to PSA immunoreactivity (Fig. 6).

In SDS-PAGE, PSA-C, -D, and -E showed single bands under nonreducing conditions (Fig. 3, left), but under reducing conditions PSA-C showed five bands in the size range 6–33 kDa, PSA-D seven bands, and PSA-E three bands (Fig. 3, right). From the staining intensities of the 33-kDa band, we estimated that peaks C, D, and E contained ~20%, 10%, and 5% of intact PSA, respectively (Fig. 3, right). All bands could be detected by immunoblotting with a polyclonal antibody to PSA (not shown). In IEF, PSA-C and PSA-D had the same pl values as PSA-B, ~6.9, whereas the pl of PSA-E, ~6.7, was clearly lower (Fig. 5, left). After neuraminidase treatment (Fig. 5, right), the pl of PSA-C increased to 7.4, and that of PSA-D and -E increased to 7.2.

Table 2 shows the amino-terminal sequences of the fragments from PSA-C, -D, and -E separated by SDS-PAGE under reducing conditions. Three different amino-terminal sequences were identified. In addition to the amino-terminus of PSA (Ile-Val-Gly-Gly-), the sequences Phe-Lys-Arg-Pro- (corresponding to residues 86–89 of intact PSA) and Lys-Leu-Gln-Cys- (corresponding to residues 146–149) (1, 23) were identified. In PSA-D, a 6-kDa band was occasionally present. The amino-terminus of this fragment was not sequenced, but it probably corresponds to a fragment derived from cleavage between residues K-185 and S-186 as reported by Watt et al. (9), which also results in a 26–28-kDa fragment with the amino-terminus Ile-Val-Gly-Gly- (Table 2, peak D).

Enzymatic Activity of PSA

PSA-A and -B showed similar enzyme activity against the chymotrypsin-restricted chromogenic peptide substrate, S-2586. This activity is only ~0.1% of that obtained with bovine chymotrypsin. PSA-C, -D, and -E displayed 5–20% of the enzymatic activity against S-2586 shown by PSA-B (Fig. 7A). Moreover, 50 μg of purified PSA from any of the peaks did not show detectable enzymatic activity against the trypsin-
restricted peptide substrate S-2222, whereas 5 ng of bovine trypsin is readily detectable with this substrate (Fig. 7B).

Complex Formation Between PSA and ACT

We added 50 pmol of either PSA-A, -B, -C, -D, or -E to a 10-fold molar excess of partially purified ACT at a final concentration of 50 nmol/L, then incubated the resulting mixtures for 48 h at 37 °C. The concentrations of PSA and PSA-ACT complex were analyzed by IFMA. About 80% of PSA-A and -B, 20% of PSA-C, 10% of PSA-D, and <5% of PSA-E complexed with ACT (Fig. 8).

Discussion

Several procedures for purification of PSA from human prostatic tissue or seminal fluid have been reported (1, 3, 9, 24, 25). Most of these are time-consuming, and the recovery has usually been only 10–15%. Of the purified PSA, only ~65% or less has been enzymatically active, as evidenced by the ability to form complexes with ACT (10). The present purification procedure is rapid and has a high yield of homogeneous PSA without use of denaturing conditions. From 30 mL of seminal fluid, we obtained 7–10 mg of intact PSA (PSA-B), corresponding to a recovery of ~30%, within 2 days.

PSA-A and -B are intact as evidenced by SDS-PAGE under reducing and nonreducing conditions, amino-terminal sequencing, and reversed-phase chromatography. These isoenzymes are also highly active enzymatically, and about 80% could form a complex with ACT. PSA-C, -D, and -E are nicked and have reduced enzymatic activity. The specific absorbance at 280 nm of thoroughly dried PSA-B at a concentration of 1 g/L was 1.61. This is higher than the value of 1.42 reported earlier (26), but lower than the theoretical value of 1.84, calculated on the basis of the amino acid and carbohydrate composition (27). Because PSA is a glycoprotein, tightly bound water can cause overestimation of weight and underestimation of the specific absorbance. However, the error is probably small, because PSA is thought to contain only one carbohydrate chain (23), and thorough drying had a very small effect on the weight of PSA.

Previous studies have shown that PSA in seminal fluid displays charge heterogeneity, with pI values from 6.8 to 8.0, mainly because of a variable degree of glycosylation (8). The component eluting first in anion-exchange chromatography (PSA-A), which comprises 5–10% of total PSA in seminal fluid, had a pI of ~7.2. This isoenzyme displayed similar enzymatic activity and molecular size in SDS-PAGE as the main isoenzyme.

Table 2. Amino-terminal sequences of PSA isoforms (A–E) obtained by anion-exchange chromatography and further separated by SDS-PAGE under reducing conditions.

<table>
<thead>
<tr>
<th>Fragment</th>
<th>PSA fraction</th>
</tr>
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<tbody>
<tr>
<td>kDa</td>
<td>A</td>
</tr>
<tr>
<td>33–34</td>
<td>IVGG*</td>
</tr>
<tr>
<td>26–28</td>
<td>IVGG</td>
</tr>
<tr>
<td>22–24</td>
<td>IVGG</td>
</tr>
<tr>
<td>18–20</td>
<td>FLRP</td>
</tr>
<tr>
<td>13–15</td>
<td>IVGG</td>
</tr>
<tr>
<td>10–12</td>
<td>KLQC</td>
</tr>
</tbody>
</table>

* The amino-terminal sequences of the individual fragments are identified by their M values. IVGG corresponds to residues 1–4, FLRP to residues 86–89, and KLQC to residues 146–149, according to cDNA sequence (23).
zyme, PSA-B, which had a pI of 6.9. After we removed sialic acid by using neuraminidase, PSA-A and -B showed the same pI (~7.7), suggesting that the difference in pI is caused by differences in glycosylation, with PSA-A containing less sialic acid than PSA-B. Thus PSA-A and -B appear to be glycosylation variants of intact PSA.

Earlier studies have shown that ~35% of PSA purified from seminal fluid displays internal peptide bond cleavage (10). By reversed-phase chromatography, Watt et al. (9) partially separated and identified three nicked forms of PSA, with nicking sites between R-85 and F-86, K-148 and K-149, and K-185 and S-186. However, Watt et al. found three more amino acid residues than Lundwall and Lilja (23) did by cDNA sequencing. Thus amino acid residues K-148–K-149 of Watt et al. correspond to K-145–K-146, and K-185–S-186 correspond to K-182–S-183, according to the cDNA sequence (23).

Our results show that the nicked forms of PSA can be separated from intact PSA by anion-exchange chromatography. This method also facilitates preparation of intact PSA in high yield. Because of disulfide bonding, nicked PSA has the same molecular size as intact PSA in SDS-PAGE under nonreducing conditions; after reduction, however, the fragments can be dissociated and separated by SDS-PAGE (Fig. 3, lanes C–E, both panels). The nicked PSA (PSA-C, -D, and -E) possessed very low or no enzymatic activity, so the slight residual activity remaining in PSA-C, -D, and -E could be due to contamination with intact PSA (PSA-A and -B). This result is compatible with the pattern in SDS-PAGE (Fig. 3, right). In spite of the different behavior in ion-exchange chromatography, the pI of PSA-C and -D was similar to that of PSA-B, whereas the pI of PSA-E was clearly lower. Sialidase treatment increased the pI values of all the forms of PSA (Fig. 5, right), but nicked PSA (PSA-C, -D, and -E) still had lower pI values than intact PSA (PSA-A and -B). This suggests that nicking itself causes a reduction in pI or that nicking is associated with further degradation of PSA.

PSA is a member of the human glandular kallikrein superfamily. It shares ~82% sequence homogeneity with the putative gene product of human kallikrein-2 (hK-2, previously called human glandular kallikrein one, hGK-1) (4). Predictions based on the DNA sequence suggest that hK-2 may have trypsin-restricted enzymatic activity (28, 29). Earlier studies have shown that purified PSA preparations may contain some trypsin-like activity (9, 10) that can be removed by affinity chromatography on aprotinin–Sepharose (10). PSA purified by the present method does not contain
trypsin-like activity at detectable amounts. This suggests that PSA purified by our method is not contaminated by hK-2.

A new and essential step in the present purification method is hydrophobic interaction chromatography, which provides about sixfold purification. Thus, after ammonium sulfate precipitation, hydrophobic chromatography, and gel filtration, but before anion-exchange chromatography, PSA is already ~94% pure. The anion-exchange chromatography step enables separation of the various isoforms of PSA. Interestingly, this step separates nicked and intact PSA, although no change in pI is expected as a result of nicking and no pI difference was actually observed between PSA-B, -C, and -D.

The structure of free PSA in circulation is not known, but at least part of it may be expected to be nicked, because it has not formed complexes with ACT or α2-macroglobulin. Preliminary results suggest that our separation method also enables us to compare the various immunoreactive forms of PSA in serum with those occurring in seminal plasma.

In conclusion, we present a rapid procedure with high yield for purification of PSA from human seminal fluid. In the last purification step, five isoforms of PSA are obtained—two intact isoenzymes and three nicked forms of PSA. The intact isoenzymes (PSA-A and -B) display high enzyme activity, and ~80% of this forms a complex with ACT. The ability to separate the various forms of PSA without the use of denaturing conditions facilitates preparation of PSA variants for standardization purposes.

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References