Prostate-Specific Antigen in Serum Occurs Predominantly in Complex with α1-Antichymotrypsin

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Immunologic measurements of the serum concentration of prostate-specific antigen (PSA), an abundant prostatic-secreted serine proteinase, are frequently used to monitor patients with prostate cancer, though it has not been ascertained whether this immunoreactivity represents a PSA zymogen, the active proteinase, or PSA complexed to extracellular proteinase inhibitors. To characterize the PSA immunoreactivity in serum, we used monoclonal antibodies produced against PSA and a polyclonal rabbit IgG against α1-antichymotrypsin in the design of three noncompetitive PSA assays: assay T, which detected PSA both when present as the active proteinase and when complexed to α1-antichymotrypsin; assay F, which recognized the active proteinase but most poorly detected PSA complexed to α1-antichymotrypsin; and assay C, which was specific for PSA complexed to α1-antichymotrypsin. We used the three assays to measure PSA immunoreactivity in 64 patients' sera and in the effluent after gel chromatography of sera from four patients. This identified an 80- to 90-kDa complex between PSA and α1-antichymotrypsin as the predominant fraction of the PSA immunoreactivity in blood plasma; an immunoreactive 25- to 40-kDa compound was the minor fraction.

Additional Keyphrases: monoclonal antibodies · prostate disease · chromatography, gel filtration · fluorimmunoassay

Prostate-specific antigen (PSA), one of the three most abundant prostatic-secreted proteins in human semen (1), is involved in the dissolution of the seminal gel structure (formed at the ejaculatory mixing of the secretions from the accessory sex glands) that occurs concomitantly with the limited proteolytic fragmentation by PSA of the predominant proteins secreted by the seminal vesicles (2-7). The single-chain 33-kDa glycoprotein, PSA, was first described by Wang et al. in 1979 (8). Independently and almost simultaneously, an apparently identical protein was discovered by Hara and Kimura (9) and Graves et al. (10). PSA was later shown to be produced as a presumably inactive precursor (11) that is converted into an active serine proteinase (3, 12).

The active proteinase has a 237-amino-acid polypeptide backbone that manifests extensive similarity with that of the glandular kallikreins (EC 3.4.21.35) (11-13) although, unlike the trypsin-like glandular kallikreins, PSA displays chymotrypsin-like substrate specificity (7, 14, 15).

In 1980, Papsidero et al. (16) were the first to report that PSA is regularly detected in the sera of prostate cancer patients. Measurements of the serum concentration of PSA are now widely used to monitor patients with prostate cancer (17, 18), although above-normal serum concentrations of PSA have also been reported both in benign prostatic hyperplasia and secondary to surgical trauma of the prostate (17, 18). However, the molecular mass of the immunoreactive form of PSA in serum is uncertain, the data reported hitherto being in disagreement as to the size of the molecules carrying the immunoreactive sites (16, 19). It has not been thoroughly investigated whether the estimated immunoreactivity represents the PSA zymogen, the active proteinase, or PSA inactivated by complex formation with extracellular inhibitors of serine proteinases. However, release of the active proteinase in intercellular fluids or blood plasma would be expected to result in rapid inactivation of the enzymatic activity by extracellular inhibitors of serine proteinases. We recently investigated the ability of PSA to react with extracellular serine proteinase inhibitors and found that purified PSA formed stable complexes with isolated α1-antichymotrypsin, α2-macroglobulin, and the α2-macroglobulin-analog pregnancy zone protein (18). This also occurred when purified PSA was added to blood plasma in vitro (15). Therefore, for the present investigation, we designed sensitive methods for the assay of noncomplexed PSA and of PSA complexed to the serine proteinase inhibitor, to characterize the molecular forms of PSA that occur in extracellular fluids in vivo.

Materials and Methods

Reagents

Molecular mass markers for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Sephacryl S-300, CNBr-activated Sepharose, and Protein A-Sepharose were from Pharmacia LKB Biotechnology (Bromma, Sweden). The Arcus 1230 fluorometer, the europium chelate of isoiohycyanatobenzylidylenetriaminetetraacetic acid, Delfia® assay buffer, and Delfia enhancement solution were from Pharmacia Wallac (Turku, Finland), and 8 × 12 microtiter strip wells were from EF-Lab (Helsinki, Finland). The ProtoBlot® sys-

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5 Nonstandard abbreviations: PSA, prostate-specific antigen; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; and Mab, monoclonal antibody.

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tem with alkaline phosphatase-conjugated goat anti-rabbit IgG and goat anti-mouse IgG was from Promega Biotec (Madison, WI). The goat anti-mouse immunoglobulin (G.A.M; heavy + light), the peroxidase-labeled isotype-specific rabbit anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgA, and IgM were from Zymed Labs., Inc. (San Francisco, CA). KC-2000 cell culture medium was from Hazleton Biologics, Inc. (Lenexa, KS), and HAT media supplement H-0262 (hypoxanthine, aminopterin, and thymidine) was from Sigma Chemical Co. (St. Louis, MO). A BioSil TSK 250 HPLC column and nitrocellulose membranes (Trans-Blot® transfer medium, 0.45-μm pore size) were from Bio-Rad Labs., Richmond, CA. Affinity-purified polyclonal rabbit IgG against PSA was produced at our laboratory as previously described (1); polyclonal rabbit IgG against α1-antichymotrypsin and rabbit anti-mouse IgG were from Dakopatt (Copenhagen, Denmark). Purified α2-macroglobulin and α1-antichymotrypsin were obtained as previously described (15). Purified PSA, consisting of ~65% active proteinase and 35% modified inactive PSA, was obtained from human semen as previously described (15). The modified inactive PSA, which contains an internal peptide bond cleavage, was purified from incubation mixtures with purified PSA and purified α1-antichymotrypsin, and isolated as previously described (15). Previously described procedures were also used to obtain stable complexes between purified PSA and purified α1-antichymotrypsin, and for two-step chromatographic isolation of complexes between PSA and α1-antichymotrypsin (15). Affinity-purified antibodies against α2-macroglobulin (obtained from a polyclonal rabbit antiseraum by affinity-chromatographic adsorption to purified α2-macroglobulin immobilized to Sepharose 4B) were immobilized to CNBr-activated Sepharose 4B with standard procedures.

The serum samples were frozen blood sera (n = 64) sent to our laboratory for routine clinical chemical analysis of prostatic acid phosphatase activity. Seminal plasma was obtained from healthy men as described previously (4) and stored at −20 °C until use.

Procedures

α2-Macroglobulin-depleted serum. Human serum, obtained from healthy women volunteers, was chromatographed on a column with immobilized rabbit IgG against α2-macroglobulin. Fractions eluting in the void volume were pooled and found by electroimmunoassay (20) to contain <1% of the initial concentration of α2-macroglobulin. This pool was stored at −20 °C until use.

Hydroxylamine-treated serum. The proteinase inhibitory activity of the α2-macroglobulin fraction in serum was irreversibly inactivated as a result of hydrolysis of its internal thioester bonds by incubating human serum from healthy women overnight at 37 °C with hydroxylamine at a final concentration of 25 mmol/L, as described by Chen et al. (21).

General procedures for fluorometric assays. Using a previously described procedure (22), we coated microtiter strip wells with 1–2 μg of IgG in 200 μL of buffer. The coated plate was stored at 4 °C and the coating was stable for more than six months. Purified PSA, monoclonal antibodies against PSA (anti-PSA Mabs), and polyclonal rabbit IgG against α1-antichymotrypsin were labeled to a specific activity of 2–5 Eu ions per molecule with the Eu chelate of isothiocyanatobenzylidene-triaminnetetracetic acid as described by Hemmilä et al. (23).

Monoclonal Antibodies

Balb/c mice were immunized by intraperitoneal injection with 70 μg of PSA emulsified with Freund's complete adjuvant. Booster doses of 50 μg were given at three- to four-week intervals and a final 40-μg booster after another three-week interval. Four days after the final booster dose, the mice were killed and their splenic lymphoid cells fused with plasmacytoma cells NS-1 at a 1:1 ratio (24, 25). The fused cells were harvested in microtiter strip wells in KC-2000 medium containing fetal calf serum, 200 mL/L, and HAT supplement (diluted 50-fold).

Anti-PSA-specific antibody production was assayed with microtiter strip wells coated with rabbit anti-mouse immunoglobulin (22). The coated strips were incubated overnight at 4 °C with hybridoma supernates, or with a Mab against PSA (0812; Hybritech Inc., San Diego, CA) used as standard and diluted in Delfia assay buffer (22); washed six times; and incubated with Eu-labeled PSA (50 ng/well) for 1 h at room temperature. The amount of bound Eu-labeled tracer was determined with an Arcus fluorometer after another six washings and the addition of Delfia enhancement solution (26).

Master cell lines were cloned by limited dilution (27). The desired cell lines were expanded intraperitoneally in Balb/c mice and the ascitic fluid was collected within 10 days. The Mab were purified by chromatography on Protein A–Sepharose according to the protocol recommended by the manufacturer.

Characterization of purified Mabs against PSA. The isotype of each Mab was determined in microtiter strip wells coated with goat anti-mouse immunoglobulin (G, A, M; heavy + light) and identified with peroxidase-labeled isotype-specific rabbit anti-mouse immunoglobulin. The ability of each antibody to recognize PSA in different PSA-containing samples was determined with sample proteins blotted to poly(vinylidene fluoride) membranes after agarose gel electrophoresis or SDS-PAGE. A previously described procedure was used to blot proteins from agarose gels (28) after electrophoresis in 75 mmol/L barbital buffer containing Ca2+, 2 mmol/L, at pH 8.6 (29). SDS–PAGE was run in gradients of 70–120 or 90–170 g/L, as described by Laemmli (30), but with the buffer system of Blobel and Dobberstein (31). Western blotting, performed essentially as described by Burnette (32), was done in an apparatus for semidry electrophoretography. Immunoreacted antibodies were detected either with alkaline phosphatase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG.

Design of noncompetitive assays. Three different as-
says were designed: assay T (total PSA), with 2E9 anti-PSA Mab as the capture antibody and Eu-labeled 2H11 anti-PSA Mab (both described below) as the detection antibody; assay F (free PSA), with the same detection antibody as assay T but with the 5A10 anti-PSA Mab (described below) as the capture antibody; and assay C (complexed PSA), with the same capture antibody as assay T but Eu-labeled polyclonal rabbit IgG against α1-antichymotrypsin as the detection antibody. Assays T and F were standardized with purified PSA (the concentration of PSA being determined by quantitative amino acid analysis), whereas assay C was standardized with an isolated complex between PSA and α1-antichymotrypsin. Samples were analyzed as follows: 25 μL of sample and 100 μL of Delfia assay buffer were incubated for 1 h in the microtiter strip wells with constant shaking, after which the wells were washed and then incubated with Eu-labeled detecting antibody (100 ng/well) for 1 h, and the amount of bound Eu-labeled antibody was determined.

Recovery of PSA added to human serum. PSA-containing samples (10–200 μg of PSA) were added in aliquots of <40 μL to 1-mL aliquots of human serum (untreated serum from healthy women volunteers, α2-macroglobulin-depleted serum, or hydroxylamine-treated serum) and incubated for 0–20 h at room temperature. After the incubation, the recovery of the added amount of PSA was measured with assay T.

Analysis for PSA in patients’ sera. PSA immunoreactivity in samples from 64 patients was measured with the three PSA assays (T, F, and C), and with the Tandem-R PSA kit (Hybritech Inc.) according to the procedure recommended by the manufacturer. Calculations of intra-assay precision and the detection limits of assays T, F, and C were made from 10 replicate analyses of the standards of each assay. The detection limit was determined with the following formula: [2 SD counts/s (std. 0)/(mean counts/s (std. 1) – mean counts/s (std. 0))] × concn (std. 1). The "clinically useful" detection limit was defined as the mean + 2 SD of the detection limit of 10 separate analyses.

Gel-filtration chromatography. Patients’ sera with very high concentrations of PSA were diluted threefold with 20 mmol/L sodium phosphate buffer (pH 6.8) containing 0.15 mol of sodium chloride per liter, and subjected to gel filtration to estimate the molecular size of the PSA-immunoreactive material. We subjected 50 μL of each diluted sample to gel filtration on a 300 × 7.5 mm Bio-Rad TSK 250 HPLC column (exclusion limit 200 kDa), equilibrated and eluted with the dilution buffer, at a flow rate of 0.5 mL/min. We collected the effluent in five-drop fractions and used Blue Dextran (2.0 MDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), and chymotrypsinogen (25 kDa) to calibrate the column. The concentration of PSA immunoreactivity in each fraction was determined with assays T, F, and C as described above.

Results
Characterization of the Epitopes Defined by Three Mabs against PSA

The three Mabs (2E9, 2H11, and 5A10) were found to be of the IgG1 isotype. Polyclonal anti-PSA IgG and the three Mabs were used to probe proteins blotted to the poly(vinylidene fluoride) membranes after agarose gel electrophoresis or SDS-PAGE (Figure 1). On blots from the agarose gel, purified PSA (lane 1, Figure 1, left) and the modified inactive PSA (not shown) were identified by the polyclonal antiserum and the three Mabs; PSA complexed to α1-antichymotrypsin was also identified by the polyclonal antiserum and by two of the Mabs but not by Mab 5A10 (lane 2, Figure 1, left).

On Western blots from SDS-PAGE of nonreduced samples, purified PSA and the modified inactive PSA were identified by the polyclonal antiserum and the three Mabs. PSA complexed to α1-antichymotrypsin was identified by the polyclonal antiserum and two of the Mabs, whereas the complex was poorly detected by the 5A10 Mab (not shown). On Western blots from SDS-PAGE of reduced samples, purified PSA (lane 1, Figure 1, right) and PSA complexed to α1-antichymotrypsin (lane 2, Figure 1, right) were identified by the 2E9 Mab and the polyclonal antiserum; the 2H11 anti-PSA Mab produced a weak, barely discernible reaction with the purified PSA (but not with the PSA complexed to α1-antichymotrypsin) (lanes 1 and 2, Figure 1C, right); the 5A10 anti-PSA Mab produced a similarly weak, barely discernible reaction with both the purified PSA and the PSA complexed to α1-antichymotrypsin (lanes 1 and 2, Figure 1D, right). In this system, the glycosylated heavy

![Figure 1](image-url)
chain (residues 1–145) in the modified inactive PSA was also identified by 2E9 Mab (not shown).

Noncompetitive Assays of PSA

Because assay T was designed to measure the total amount of PSA in a sample, both when present in its noncomplexed form and when inactivated by complex formation with α₁-antichymotrypsin, we used two Mabs (2E9 and 2H11) that identified both purified PSA and PSA complexed to α₁-antichymotrypsin in the blotting procedure from agarose gels. In assay F, designed specifically to measure the noncomplexed PSA, we used a capture antibody (5A10) that identified purified PSA but not the PSA complexed to α₁-antichymotrypsin in blots from agarose gels, and the same Eu-labeled detection antibody (2H11) as in assay T. The capture antibody in assay C (2E9), recognizing both PSA and PSA complexed to α₁-antichymotrypsin in the blots from agarose gels, was combined with the Eu-labeled polyclonal rabbit IgG against α₁-antichymotrypsin to render the assay specific for PSA complexed to α₁-antichymotrypsin.

Analysis for purified PSA and the PSA in the pooled seminal plasma gave identical dose responses in assay T. Although the dose–response of purified PSA was slightly greater than that of the purified PSA incubated with α₁-antichymotrypsin, the dose–response curve for the PSA in pooled seminal plasma was identical with that for the PSA in pooled seminal plasma incubated with α₁-antichymotrypsin in this assay (Table 1). Moreover, purified PSA, the PSA in pooled seminal plasma incubated with α₁-antichymotrypsin, and the isolated complex between PSA and α₁-antichymotrypsin all displayed identical, parallel, and linear dose–response relationships in the assay (Figure 2, left).

With assay F, purified PSA and the PSA in the pooled seminal plasma gave almost identical dose–response curves, whereas only 30–50% of the purified PSA incubated with α₁-antichymotrypsin or of the PSA in pooled seminal plasma incubated with α₁-antichymotrypsin was detected (Table 1). The detected fraction in the purified PSA incubated with α₁-antichymotrypsin and the PSA in seminal plasma incubated with α₁-antichymotrypsin is presumably the 30–40% of the PSA in the seminal plasma that contains an internal peptide bond cleavage that renders the protein unreactive with α₁-antichymotrypsin (15). Thus, the PSA complexed to α₁-antichymotrypsin appears to be poorly detected by this assay. Consistent with this interpretation, the isolated PSA–α₁-antichymotrypsin complex was very poorly detected by the assay (Table 1) and did not display any parallel dose–response relationship as compared with purified PSA (Figure 2, middle).

Assay C (with Eu-labeled polyclonal rabbit IgG against α₁-antichymotrypsin as the detecting antibody) was specifically designed to detect PSA in complex with α₁-antichymotrypsin; thus, it failed to detect either purified PSA or PSA in seminal plasma (Table 1; Figure 2, right). Therefore, assay C had to be standardized with the isolated PSA–α₁-antichymotrypsin complex, the concentration of PSA in the complex having been determined with assay T. Only 60–70% of the purified PSA incubated in α₁-antichymotrypsin or the PSA in seminal plasma incubated with α₁-antichymotrypsin was detected by the assay. This agrees with the analysis of these samples with assay F and with our previous finding that 30–40% of the PSA in these samples was unreactive with α₁-antichymotrypsin (15).

Reactions after Adding PSA to Serum

If purified PSA or pooled seminal plasma was added to serum (experiments 1 and 2, Table 2), the recovery of the added amount of PSA decreased within 1 h to 60–70% of the original value with assay T. The poor recovery of PSA in these experiments might have been due to inaccessibility of the epitopes of PSA after its complexing with α₂-macroglobulin. This complex formation is somewhat faster than that between PSA and α₁-antichymotrypsin after in vitro addition of PSA to blood serum (15). To check this finding, we added the isolated PSA–α₁-antichymotrypsin complex to serum (experiment 3, Table 2). In another experiment, the PSA in seminal plasma was allowed to form a stable complex with α₁-antichymotrypsin by incubating the seminal plasma with α₁-antichymotrypsin for 2 h before the seminal plasma reacted with α₁-antichymotrypsin was added to serum (experiment 4, Table 2). Both experiments resulted in 90–95% recovery of the added amount of PSA. The interpretation that the poor recovery of PSA is due to its complex formation with α₂-macroglobulin in blood plasma is supported by the high recovery (89–98% of the original value) of PSA when the seminal plasma was added to α₂-macroglobulin-depleted serum (experiment 5, Table 2). The same high recovery of PSA was obtained when seminal plasma was added to serum containing hydroxylamine-inactivated α₂-macroglobulin (experiment 6, Table 2).

**Table 1. Relative Responses of the Three PSA Assays**

<table>
<thead>
<tr>
<th>Sample*</th>
<th>Relative response, %</th>
<th>Assay T</th>
<th>Assay F</th>
<th>Assay C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purified PSA</td>
<td>100</td>
<td>100</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Purified PSA and 200 μg of purified α₁-antichymotrypsin</td>
<td>93</td>
<td>50</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Pooled seminal plasma</td>
<td>97</td>
<td>95</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Pooled seminal plasma and 200 μg of purified α₁-antichymotrypsin</td>
<td>98</td>
<td>35</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>1:1 molar ratio complex between purified PSA and α₁-antichymotrypsin</td>
<td>97</td>
<td>8</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

* Samples containing 25 μg of PSA were incubated for 2 h after dilution with phosphate-buffered saline (20 mmol of sodium phosphate and 150 mmol of NaCl per liter, pH 7.2), then were further diluted 200-fold with Tris-buffered saline (per liter, 50 mmol of Tris, 150 mmol of NaCl, 0.5 g of Na₂CO₃ and 75 g of bovine serum albumin, pH 7.75) before assay.

*In assays T and F, the response magnitude of each sample was expressed in percent of purified PSA, a relative response of 100% being assigned to the purified PSA. In an identical procedure, the response magnitude of each sample in assay C was expressed in percent of purified complex between PSA and α₁-antichymotrypsin, this complex having been assigned to give a relative response of 100% in assay C.
Table 2. Analytical Recovery of Different Forms of PSA Added to Human Serum

<table>
<thead>
<tr>
<th>Experiment*</th>
<th>0 h</th>
<th>1 h</th>
<th>20 h</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>109 (101)</td>
<td>64 (59)</td>
<td>not determined</td>
</tr>
<tr>
<td>2</td>
<td>27 (99)</td>
<td>17 (63)</td>
<td>not determined</td>
</tr>
<tr>
<td>3</td>
<td>145 (98)</td>
<td>101 (67)</td>
<td>not determined</td>
</tr>
<tr>
<td>4</td>
<td>71 (95)</td>
<td>53 (72)</td>
<td>not determined</td>
</tr>
<tr>
<td>5</td>
<td>215 (104)</td>
<td>197 (95)</td>
<td>190 (91)</td>
</tr>
<tr>
<td>6</td>
<td>102 (104)</td>
<td>91 (93)</td>
<td>94 (96)</td>
</tr>
<tr>
<td>7</td>
<td>10.7 (94)</td>
<td>10.3 (90)</td>
<td>9.8 (86)</td>
</tr>
<tr>
<td>8</td>
<td>182 (97)</td>
<td>185 (104)</td>
<td>179 (95)</td>
</tr>
<tr>
<td>9</td>
<td>96 (99)</td>
<td>104 (107)</td>
<td>90 (93)</td>
</tr>
<tr>
<td>10</td>
<td>11.4 (101)</td>
<td>10.5 (93)</td>
<td>10.7 (95)</td>
</tr>
<tr>
<td>11</td>
<td>182 (101)</td>
<td>154 (96)</td>
<td>143 (89)</td>
</tr>
<tr>
<td>12</td>
<td>81 (97)</td>
<td>78 (94)</td>
<td>85 (102)</td>
</tr>
<tr>
<td>13</td>
<td>8.6 (98)</td>
<td>7.8 (89)</td>
<td>7.9 (89)</td>
</tr>
<tr>
<td>14</td>
<td>161 (100)</td>
<td>149 (92)</td>
<td>150 (93)</td>
</tr>
<tr>
<td>15</td>
<td>77 (95)</td>
<td>74 (91)</td>
<td>69 (85)</td>
</tr>
<tr>
<td>16</td>
<td>8.8 (102)</td>
<td>8.2 (98)</td>
<td>8.6 (102)</td>
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</table>

*Samples of purified PSA or seminal plasma (10-200 µg of PSA) were added to serum in the following forms: exp. 1, purified PSA; exp. 2, pooled seminal plasma; exp. 3, isolated complex with α1-antichymotrypsin; exp. 4, seminal plasma (complexed to α1-antichymotrypsin by incubation of PSA in pooled seminal plasma with a fourfold molar excess of purified α1-antichymotrypsin at 37 °C for 2 h); exp. 5, pooled seminal plasma (the serum being α2-macroglobulin depleted); and exp. 6, pooled seminal plasma (the serum having been hydroxyamine treated). PSA was measured at various times after mixing.

Analysis for PSA in Serum

We measured the concentration of PSA immunoreactivity in serum samples from 64 patients, using the three assays (T, F, and C). Estimates of the intra-assay coefficients of variation were 7.2-3.4% over the range of concentrations of the standard curve with assay T, 9.4-4.0% with assay F, and 13.5-6.2% with assay C. The detection limit, calculated as described earlier, was 0.12 µg/L in assay T, 0.15 µg/L in assay F, and 0.56 µg/L in assay C. Regression analysis of the results obtained with assays T and C gave y = 0.89x + 6.55 (r = 0.97). This is consistent with the regression analysis between assays T and F: y = 0.10x + 3.56 (r = 0.82). Furthermore, the analysis with assays T and C indicated that >50% of the PSA immunoreactivity in serum (median value 86%) was complexed to α1-antichymotrypsin in 56 of the 64 patients' samples. This agrees with the finding that 7-50% of the PSA immunoreactivity in serum (median value 22%) was not in complex with α1-antichymotrypsin in 56 of the patients' sera analyzed with assays T and F. In eight of the sera, assays C and T indicated that <50% of the PSA immunoreactivity was complexed with α1-antichymotrypsin. This result also agreed with those of assays F and T, which indicated that >50% of the PSA immunoreactivity was not complexed with α1-antichymotrypsin in these sera. Regression analysis of the results obtained with assay T (x) and the Tandem-R PSA (y) gave y = 1.10x - 0.90 (r = 0.98, n = 58).

The molecular mass of the PSA immunoreactivity in sera of four patients was studied by gel-filtration chromatography, followed by analysis of the PSA immunoreactivity in the eluted fractions with each of the three assay procedures (Figure 3). The recovery of PSA immunoreactivity from the gel-filtration chromatography was 82-107%, as measured in the eluted fractions with the three different assays. Fractions analyzed with assay T, which detected noncomplexed PSA and the PSA complexed to α1-antichymotrypsin equally well, contained one predominant peak of PSA immunoreactivity that eluted at a volume corresponding to a mass of 80-90 kDa. Post-chromatographic analysis of patients' sera with assay C, which detected PSA complexed to α1-antichymotrypsin but not noncomplexed PSA, also identified one predominant peak of immunoreactivity that eluted at a volume corresponding to a mass of 80-90 kDa.

Analysis of chromatographed fractions with assay T also identified a minor peak of PSA immunoreactivity that eluted at a volume corresponding to a molecular mass of 25-40 kDa (Figure 3), a mass corresponding to that previously reported for purified PSA (8, 17, 15). The idea that this minor peak of PSA immunoreactivity may represent noncomplexed PSA derives support from the inability of assay C to identify any distinct peak of immunoreactivity eluting at this position in the chro-
molecule, as demonstrated by the inability of Mab 2H11 to detect PSA blotted to poly(vinylidene fluoride) membranes after SDS–PAGE of reduced samples.

Mab 5A10 defines a third epitope on the PSA molecule, one that is almost inaccessible when PSA is complexed to α1-antichymotrypsin. This epitope is also sensitive to conformational changes of PSA, as demonstrated by the inability of Mab 5A10 to detect PSA blotted to poly(vinylidene fluoride) membranes after SDS–PAGE of reduced samples.

We used Mabs 2E9 and 2H11 in the design of the noncompetitive assay (assay T) capable of detecting both uncomplexed PSA and PSA complexed to α1-antichymotrypsin because these antibodies define two different PSA epitopes, both of which remain accessible when PSA is complexed to α1-antichymotrypsin. The required specificity of the assay was confirmed by the most identical dose–response relationships obtained for uncomplexed PSA and for PSA complexed to α1-antichymotrypsin. Thus, the concentration of PSA in the purified complex between PSA and α1-antichymotrypsin could also be determined with assay T by using the purified PSA as standard. We therefore used this complex to standardize assay C, which, owing to the design of the assay, did not detect purified PSA.

In contrast, assay F was designed solely to detect purified PSA (and not purified PSA complexed to α1-antichymotrypsin). Thus, purified PSA and PSA in seminal plasma displayed almost identical dose–response relationships when analyzed with this assay, whereas PSA complexed to α1-antichymotrypsin was very poorly recognized. The poor recognition of this complex suggests that Mab 5A10 (the solid-phase capture antibody in assay F) defines an epitope on the PSA molecule that is poorly accessible on PSA complexed to α1-antichymotrypsin, and that Mab 2H11 defines an epitope that is accessible on PSA complexed to α1-antichymotrypsin. This interpretation is also consistent with the finding (see above) that Mab 5A10 was unable to detect PSA complexed to α1-antichymotrypsin in immunoblotting experiments.

Using assay T, in which purified PSA and PSA complexed to α1-antichymotrypsin displayed identical dose–response relationships, we obtained a low recovery of the PSA immunoreactivity when purified PSA or PSA in seminal plasma was added to blood serum in vitro. We believe the poor recovery may be due to complex formation between PSA and proteinase inhibitors other than α1-antichymotrypsin. Recently, active PSA has been reported to form SDS-stable complexes with α2-macroglobulin when added to human serum in vitro (18). The in vitro rate of complex formation between PSA and α2-macroglobulin was higher than that of PSA and α1-antichymotrypsin (18). α2-Macroglobulin is known to encapsulate the target enzyme at complex formation (33), which is consistent with the previously reported loss in PSA immunoreactivity of the PSA and α2-macroglobulin complex as analyzed by electroimmunoassay (15). The ability of α2-macroglobulin to encap-

Fig. 3. Gel chromatography of PSA immunoreactivity in serum samples

Patients’ sera (50 μL of a threefold dilution) were chromatographed on a Biosil TSK 250 HPLC column and the effluent was measured for PSA immunoreactivity with assay T (C), assay F (D), and assay C (×) (see Fig. 2 caption for description of assays). The elution positions for molecular mass references (Blue Dextran 2.0 mDa, bovine serum albumin 67 kDa, ovalbumin 43 kDa, and chymotrypsigenin 25 kDa) were determined in separate runs. PSA immunoreactivity of sample I was 1590 μg/L with assay T, 265 μg/L with assay F, and 1894 μg/L with assay C; that of sample 2 was 1134, 225, and 1225 μg/L, respectively; that of sample 3 was 705, 85, and 643 μg/L, respectively; and that of sample 4 was 1745, 90, and 1921 μg/L, respectively.

Discussion

The data presented here include a partial characterization of the epitope specificity of three Mabs against PSA. However, to facilitate interpretation of some of the findings, we emphasize that the purified PSA used in the experiments was obtained from seminal fluid, where the active serine proteinase constitutes 60–70% of the total PSA content; 30–40% is inactive because of an internal peptide bond cleavage (15). One of the Mabs against PSA (2E9) identifies an epitope on the PSA molecule still exposed on the PSA complexed to α1-antichymotrypsin. This Mab may be relatively insensitive to conformational changes of the PSA molecule because, on Western blots from SDS–PAGE, Mab 2E9 detected both the free uncomplexed PSA molecule and PSA complexed to α1-antichymotrypsin. We found this epitope to be located on the heavy chain of the cleaved inactive PSA (residues 1–145).

Mab 2H11 also defines an epitope on the PSA molecule that remains accessible when PSA is complexed to α1-antichymotrypsin. In all likelihood this epitope is different from that defined by Mab 2E9, the former being sensitive to conformational changes of the PSA
ulate a target proteinase is also consistent with our recovery experiments, where a loss of PSA immunoreactivity in blood serum was found with the in vitro addition of active PSA. This encapsulation property of α2-macroglobulin is also supported by the absence of loss in PSA immunoreactivity both when PSA was allowed to form a stable complex with α1-antichymotrypsin before being added to the serum, and when active PSA was added to α2-macroglobulin-depleted serum or to serum with inactivated α2-macroglobulin. These performance data may also be interpreted as evidence that assay T is unable to detect PSA complexed to the α2-macroglobulin, a conclusion also strongly supported by the close correlation of assays T and C in the analyses of 64 patients’ sera.

The findings from the assay of PSA immunoreactivity in patients’ sera strongly suggest that PSA complexed to α1-antichymotrypsin constitutes the predominant form of immunoreactive PSA in blood plasma in vivo. Evidence of this is based on both the analysis of the molecular mass of the PSA immunoreactivity in sera of four patients determined with assay T (which detects both noncomplexed PSA and PSA complexed to α1-antichymotrypsin) and with assay C (which solely detects PSA complexed to α1-antichymotrypsin), and the close correlation between results obtained with these two assays in the analyses of 64 patients’ sera. Moreover, the results obtained for patients’ sera with assay T correlated very closely to those obtained with the Tandem-R PSA kit, which suggests that the Tandem-R PSA assay also detects PSA complexed to α1-antichymotrypsin as the predominant form of the immunoreactive PSA in patients’ sera. The reported half-life of PSA immunoreactivity in serum—two to three days (34, 35)—is more compatible with that expected for the inactive complex between PSA and α1-antichymotrypsin than the short half-life expected for an active 33-kDa proteinase in vivo.

The results of analysis for the different forms of PSA immunoreactivity in patients’ sera also indicate that free noncomplexed PSA is a minor fraction in serum. This conclusion is based on estimations of the molecular mass of the PSA immunoreactivity in patients’ sera, where assay T identified only a minor immunoreactive 25- to 40-kDa fraction, a fraction constituting <30% of the total PSA immunoreactivity in four patients’ sera as also analyzed with assay T. At an identical position in the chromatogram, assay F (which detects the free noncomplexed PSA but poorly detects the PSA complexed to α1-antichymotrypsin) identified the major fraction of the PSA immunoreactivity in the same sera. That the 25- to 40-kDa free (noncomplexed) PSA immunoreactivity is a minor fraction of the total anti-PSA immunoreactive proteins present in the 64 patients’ sera is also supported by our comparison of the results of the analyses performed with assays T and F. The findings provide no indication of whether the noncomplexed 25- to 40-kDa PSA immunoreactivity constitutes the PSA zymogen, active PSA, or the PSA inactivated as a result of an internal proteolytic cleavage such as that demonstrated in a minor fraction of the PSA in seminal fluid (15). This issue will require further study.

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