Characterization of Novel Monoclonal Antibodies for Prostate-Specific Antigen (PSA) with Potency to Recognize PSA Bound to $\alpha_2$-Macroglobulin

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Background: Different molecular forms of prostate-specific antigen (PSA) have been used to differentiate between benign prostatic hyperplasia and prostate cancer. Detecting PSA bound to endogenous inhibitors such as $\alpha_1$-antichymotrypsin (ACT) and $\alpha_2$-macroglobulin ($\alpha_2$M) is often difficult because of epitope masking or sensitivity problems. Here we report the characterization of four novel mouse monoclonal antibodies (mabs) obtained by immunization with PSA-$\alpha_2$M complexes. Their ability to detect free PSA and PSA-inhibitor complexes was shown, and their epitopes were analyzed by phage display technology.

Methods: The properties of the mabs were studied by competition and sandwich assays and by Western blotting. Epitope mapping was performed by screening of a phage display peptide library.

Results: All four mabs recognized free PSA, PSA-ACT, and PSA-$\alpha_2$M complexes, but to various degrees. With different combinations of mabs in competition experiments, antibodies were identified that enhance binding of other mabs to PSA, forming the molecular basis of a very sensitive assay for the detection of PSA and PSA-ACT complexes. Mabs with highest reactivity for PSA-$\alpha_2$M were selected to establish an immunoassay for that complex. Western blot analysis revealed that all mabs recognized conformational epitopes of PSA. These findings were supported by phage display results demonstrating mimotopes in the PSA molecule.

Conclusion: The results presented here could aid in the further development of clinically relevant assays for PSA and PSA-$\alpha_2$M complexes. 
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Serum prostate-specific antigen (PSA)\(^1\) appears to be an important marker for the diagnosis and management of prostate cancer. Unfortunately, the diagnostic specificity of total PSA in serum, particularly at concentrations of 2.5–10 $\mu$g/L, for the detection of prostate cancer is low. Increased PSA is found in patients with benign prostatic hyperplasia or bacterial prostatitis and after prostatic manipulations and trauma.

PSA exists in serum in different molecular forms that can be measured separately and contribute to differentiation between benign prostatic hyperplasia and prostate cancer. It forms stable complexes with several extracellular protease inhibitors, predominantly $\alpha_1$-antichymotrypsin (ACT) and $\alpha_2$-macroglobulin ($\alpha_2$M). Smaller amounts bind to protein C inhibitor and $\alpha_1$-antitrypsin. PSA-ACT is the predominant immunoreactive form in serum and accounts for 70–80% of the immunoreactivity (1, 2). A smaller fraction, 20–30%, of PSA remains unbound in serum in spite of the 10$^5$- to 10$^6$-fold molar excess of protease inhibitors such as $\alpha_2$M. In vitro, $\alpha_2$M rapidly forms complexes with PSA, which cannot be detected by commercial immunoassays because PSA is enveloped by the 720-kDa $\alpha_2$M molecule during complex formation. However, PSA complexes can be rendered immunoreactive by denaturation with sodium dodecyl sulfate (SDS)
(3) or at high pH (4), which forms the basis for measuring liberated PSA by ELISA. PSA immunoassays are widely used to detect early-stage prostate cancer, to evaluate disease progression, and to assess therapeutic response. Furthermore, PSA concentrations may be used to identify postsurgical residual disease or tumor recurrence (5). In addition to the total serum PSA concentration (free PSA plus PSA-ACT), the ratio of free to total PSA has become an important variable for distinguishing between males with benign and malignant prostatic disease (6). Because PSA-α2M complexes have the fastest elimination pattern as a result of rapid endocytosis via the α2M receptor (LRP1 or CD91), it could be assumed that this currently heavily detectable complex may play an important role in PSA regulation and metabolism. It therefore could be of potential benefit in differentiating between benign and malignant prostatic disease. Difficulties in detecting this complex by common immunoassays are the reason that evaluation of the PSA-α2M complex is not part of the prostate cancer diagnostic program.

The purpose of the present study was to produce and characterize new monoclonal anti-PSA antibodies, to examine their epitope configurations, and to test their practicability for the development of sensitive immunoassays that can differentiate serologic forms of PSA.

### Materials and Methods

#### Biological Materials

PSA was purified from seminal fluid according to the method described by Lilja (7). Complexes between PSA and ACT or α2M were prepared as described recently (8). The concentrations of the PSA complexes were determined by a modified Bradford method (9). Native α2M, anti-CD91, anti-α2M monoclonal antibody (mab; clone α-11), and horseradish peroxidase (HRP)- and biotin-labeled anti-α2M immunoglobulins were obtained from BioMac GmbH. The Immulite assay from DPC was applied to analyze free PSA and PSA-ACT in solutions. HRP-labeled streptavidin and HRP-labeled polyclonal anti-mouse IgG were from Dako.

#### MAB Generation

Female Balb/c mice were immunized by intraperitoneal injection with 50 μg of freshly prepared PSA-α2M complex emulsified with an equal volume of Freund’s adjuvant, followed by three booster injections containing 20 μg of antigen in incomplete adjuvant in the 6th and 8th weeks after initial injection. Two weeks later, the mice received injections containing 20 μg of PSA-α2M without adjuvant, and spleen cells were obtained the following day. The spleen cells were fused with SP2/0-Ag14 mouse myeloma cells and propagated according to the standard procedure described by Köhler and Milstein (10). Selected clones were cultured in a MiniPerm culture system (Greiner-Bio One), and the mabs were prepared from the supernatant by protein A-Sepharose chromatography.

Purified monoclonal or polyclonal antibodies were conjugated to HRP with use of preactivated HRP from Boehringer.

#### ELISA

Different ELISA conditions were used. Briefly, PSA or PSA-inhibitor complex (2 mg/L in 0.1 mol/L carbonate buffer, pH 9.1) was added to the wells of 96-well plates (NUNC) and incubated at 4 °C overnight. After three washing steps with phosphate-buffered saline–Tween (PBS-T; 50 mmol/L sodium phosphate, 150 mmol/L sodium chloride, 0.5 mL/L Tween-20, pH 7.4), mabs dissolved in the washing buffer were added to each well (200 μL) and incubated for 1 h at 37 °C. After three additional washing steps, bound mabs were detected by incubation with 200 μL of HRP-conjugated polyclonal rabbit anti-mouse IgG (dilated 1:1000) in PBS-T for 1 h at 37 °C and development with o-phenylenediamine/H₂O₂. One row of wells was used as blank containing immobilized PSA but no mab.

In a separate setting, a sandwich ELISA was prepared as follows: 96-well plates were coated with 200 μL of anti-PSA mab (2 mg/L in 0.1 mol/L carbonate buffer, pH 9.1) overnight at 4 °C. The plates were then washed as described, and 200 μL of a solution containing PSA, PSA-ACT, or PSA-α2M diluted in PBS-T to various concentrations was added. After incubation for 1 h at 37 °C, plates were washed three times, and 200 μL of HRP-conjugated anti-PSA mab or anti-α2M IgG (1:1000) dissolved in PBS-T was added and incubated for 1 h at 37 °C. Color development was performed as described above. Again, the blank containing the immobilized mab but no antigen was used to determine nonspecific adsorption.

In a third experimental setting, a biotin-labeled polyclonal anti-α2M IgG (1:1000) instead of anti-α2M IgG was added and incubated for 1 h at 37 °C. After three additional washing steps, 100 μL of Eu³⁺-labeled streptavidin (Perkin-Elmer) dissolved in PBS containing 1 g/L bovine serum albumin was added and incubated for 30 min at 4 °C. After washing, 100 μL of enhancement solution was added and incubated for 10 min at room temperature. Absorbance was measured at 615 nm by time-resolved fluorometry.

#### Competition Studies

ELISA plates were coated with 200 μL of PSA solution (2 mg/L in 0.1 mol/L carbonate buffer, pH 9.1) at 4 °C overnight. Plates were washed three times, and 200 μL of HRP-labeled PSA-C1 (diluted 1:1000) containing increasing concentrations of competitive unconjugated anti-PSA mabs was added. After incubation for 1 h at 37 °C, plates were washed three times, and the bound HRP-labeled PSA-C1 mab was detected by o-phenylenediamine/H₂O₂.

#### Western Blotting

PSA (3 μg) was incubated with SDS or SDS/β-mercaptoethanol, respectively, and boiled for 5 min. SDS–polyac-
rylamide gel electrophoresis (SDS-PAGE) and Western blotting were performed as described previously by Otto et al. (8). For semiquantitative analysis of colored bands, the Herolab E.A.S. Yplus-System was used.

**PHAGE DISPLAY**

For screening the mab epitopes, we used a heptapeptide phage display library (Ver. 1.01; New England BioLabs) containing 10⁹ possible linear 7-residue sequences. Mabs (200 µL) were coated on plastic tubes (100 mg/L in 0.1 mol/L NaHCO₃, pH 8.6) overnight. After three washing steps, the tubes were incubated with 2 × 10¹¹ phages. After three rounds of biopanning, the bound phages were identified and amplified, and their DNA was isolated. After DNA sequencing, the corresponding peptides were deduced.

**MEASUREMENT OF PSA ACTIVITY**

PSA protease activity was measured by use of the chromogenic substrate N-succinyl-Ala-Ala-Pro-Phe p-nitroanilide (cat. no. S7388; Sigma-Aldrich). The reaction mixture (1 mL) contained 0.1 mol/L Tris-HCl (pH 7.8), 0.1 mol/L CaCl₂, and 1 mmol/L substrate. After addition of PSA (20 µg), the samples were incubated at 37 °C for 60 min, and the absorbance was measured at 405 nm. Mabs were included in the incubation mixture in twofold excess to PSA to assess their effect on the proteolytic activity of the enzyme.

**Results**

**REACTIVITY MEASUREMENT**

PSA-α₂M complexes were used for immunization of mice. It is known that antigens, when bound to α₂M, elicit a strong immune response. This is the result of uptake of α₂M-antigen complexes by the α₂M receptor (LRP1 or CD91), which is present on antigen-presenting cells such as dendritic cells or macrophages (11). Of 30 clones, 4 (PSA-C1, PSA-C2, PSA-C3, and PSA-C4) produced highly reactive mabs to PSA.

These mabs showed high affinity to PSA and did not exhibit reactivity with PSA-negative human serum. They were further characterized according their ability to bind PSA and its complexes in ELISA experiments under various conditions, i.e., recognition of antigens in solution or after antigen immobilization, respectively.

PSA, PSA-ACT, and PSA-α₂M were immobilized on 96-well plates, and increasing dilutions of different mabs (stock solutions adjusted to 1 g/L) were added and allowed to react with the immobilized antigen. Detection was achieved by use of HRP-labeled polyclonal anti-mouse IgG. That dilution producing a signal that corresponded to the blank mean + 2 SD was defined as the reactivity equivalent, corresponding to the detection limit or sensitivity. As shown in Table 1, two mabs, PSA-C1 and PSA-C4, were able to detect all three PSA forms, whereas PSA-C3 and PSA-C2 detected primarily free PSA and had very low reactivity toward PSA-ACT or PSA-α₂M.

To exclude the possibility that the mabs may be reactive with traces of free PSA that were present in PSA-ACT and PSA-α₂M preparations, we analyzed traces of free PSA with the commercial Immulite immunoassay. The respective concentration of PSA measured by this assay was then used for coating ELISA plates. We could show that all four mabs were unable to detect these traces of free PSA and were therefore confident that the detection signals produced in the PSA-ACT and PSA-α₂M assays were caused only by PSA bound to the inhibitors.

**ANTIBODY Pairing BY COMPETITIVE BINDING TO IMMobilized PSA**

Mabs conjugated with HRP were allowed to react with immobilized PSA in the presence of increasing concentrations of nonconjugated mabs to demonstrate the ability of any mab to block its own binding and that of other mabs. Representative competition analysis using HRP-

<table>
<thead>
<tr>
<th>Mab</th>
<th>Detection limit for PSA</th>
<th>Detection limit for PSA-ACT</th>
<th>Detection limit for PSA-α₂M</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSA-C1</td>
<td>1:512 000</td>
<td>1:256 000</td>
<td>1:64 000</td>
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<tr>
<td>PSA-C4</td>
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<td>PSA-C3</td>
<td>1:512 000</td>
<td>1:1000</td>
<td>1:1000</td>
</tr>
<tr>
<td>PSA-C2</td>
<td>1:128 000</td>
<td>1:1000</td>
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</tr>
</tbody>
</table>

* Purified PSA, PSA-ACT, and PSA-α₂M were immobilized in the wells of 96-well plates (2 mg/L). Respective mabs (stock solution of 1 g/L) were added in decreasing concentrations to the wells and incubated for 1 h at 37 °C. After washing, the bound mabs were detected by incubation with HRP-labeled polyclonal anti-mouse IgG (200 µL; 1:1000 dilution) for 1 h at 37 °C. The detection limit was defined as the mean + 2 SD for the blank.

Fig. 1. Competition of mabs for binding to immobilized PSA. We coated the wells of 96-well plates with PSA (2 mg/L) and incubated them, in the presence of constant amounts of HRP-labeled PSA-C1 (1:10000 dilution), with increasing amounts of PSA-C3, PSA-C4, PSA-C2, PSA-C1, and an unrelated mab (α-11) that recognizes human α₂M.
labeled PSA-C1 as the detection antibody is shown in Fig. 1. As expected, the detection antibody competed with the unlabeled PSA-C1. PSA-C2 was found to partially inhibit binding of the detection mab to immobilized PSA. Surprisingly, in the presence of PSA-C4 and PSA-C3, the binding of PSA-C1 to PSA was highly increased, rendering the detection of PSA more sensitive. As expected, an unrelated mab, α-11, which recognizes human α2M, did not affect binding of the detection mab to PSA.

SANDWICH ASSAYS

On the basis of the results of competition studies, we tested all combinations of the mabs in a sandwich assay for PSA and PSA-ACT. The most effective combinations of mabs are shown in Table 2, excluding the combination of PSA-C2 and PSA-C1 for the reasons mentioned above.

The mab pair of immobilized PSA-C1 and PSA-C4-HRP could detect PSA with high sensitivity: <10 ng/L of the antigen could be measured. This assay was found to recognize free PSA and PSA-ACT complexes with an equimolarity index of 85% (100% represents equal absorbance for both antigens at a given concentration; Fig. 2). It should be mentioned that the calculation is based on the total protein content of the antigens. In the case of PSA-ACT, we would expect a left-shift of the curve for calculations made based on the PSA content. However, because of conformity, this was omitted because the amount of protein used for the immobilization experiments shown in Table 1 was calculated on the same basis. The immobilized PSA-C2 in combination with PSA-C4-HRP was found to detect almost equimolar amounts of PSA and PSA-ACT but with less sensitivity than the combination of PSA-C1 and PSA-C4. As far as the detection of PSA is concerned, this can be expected from the lower reactivity of PSA-C2 with free PSA (Table 1). Unexpectedly, we obtained a low sensitivity for free PSA when we used the mab pair PSA-C3 and PSA-C1-HRP (Fig. 3). This is quite surprising because the binding properties of this combination resemble those of the pair PSA-C1 and PSA-C4 (Fig. 1 and Table 1). These discrepancies might be caused by differences in antigen recognition depending on the mode of antigen presentation, e.g., adsorbed to a plastic surface or free in solution. This might also explain why PSA-ACT is fairly detectable in the sandwich assay (Fig. 2 and Fig. 3) compared with its low detectability after being immobilized to the microtiter plate (Table 1).

When assessing the reactivity of the different combinations of mabs toward PSA-α2M complexes in buffer solution, none of these settings could detect the complex

<table>
<thead>
<tr>
<th>Immobilized antibody</th>
<th>Optimal binding partner</th>
<th>Detection limit for PSA, µg/L</th>
<th>Detection limit for PSA-ACT, µg/L</th>
<th>Equimolarity, %</th>
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<td>PSA-C4-HRP</td>
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<td>70</td>
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<tr>
<td>PSA-C4</td>
<td>PSA-C1-HRP</td>
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<td>0.065</td>
<td>50</td>
</tr>
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</table>

*Different mabs were immobilized in the wells of 96-well plates overnight (2 mg/L). After washing, 200 µL each of PSA and PSA-ACT (stock solutions of 1 g/L were used) were added in decreasing concentrations (serial dilution) and incubated for 1 h at 37 °C. The bound PSA and PSA-ACT was detected with a defined HRP-labeled mab. The detection limit was defined as the lowest concentration of PSA or PSA-ACT that produced a signal greater than the mean for the blank +2 SD. The "equimolarity" of detection is given in percentage by comparing the detection signal of various PSA concentrations with those gained from PSA-ACT measurements.
at acceptable rates. This is obviously a result of the steric hindrance produced when two mabs are bound simultaneously to caged PSA. We therefore decided to use anti-PSA mabs as capture antibodies and a polyclonal HRP-labeled anti-α₂M antibody as the detection antibody. When we used this combination, the lower limits of quantification for the PSA-α₂M complex were 37.5 and 41 μg/L, respectively, for PSA-C4 and PSA-C1, whereas no signal was produced with PSA-C2 and PSA-C3 in that range (Fig. 4). This calculation was based on the assumption of a 1:1 PSA-α₂M ratio (8). As already indicated in Table 1, these results demonstrated that PSA is not totally embedded in the cavity of α₂M and has exposed epitopes.

Detection was substantially improved when we used biotin-labeled polyclonal anti-α₂M antibody in combination with Eu³⁺-labeled streptavidin in time-resolved fluorometry. It was possible to detect PSA-α₂M complex concentrations as low as 10 μg/L when we used PSA-C1 as capture antibody.

To analyze the ability of the mabs to detect PSA-α₂M complexes in whole serum, we added 50 or 100 μg/L purified PSA-α₂M complex to four aliquots of serum from a female whose PSA concentration was 77 g/L (82%) and 77 (11) μg/L (77%) of the complex, respectively. The fact that there was incomplete recovery indicates a minor interference with other serum components.

**WESTERN BLOTTING**

SDS-PAGE under nonreducing and reducing conditions followed by Western blotting revealed that none of the four mabs detected PSA under reducing conditions (results not shown). This indicates that all mabs recognize exclusively conformational epitopes in the PSA molecule.

**INHIBITION BY MABS OF BINDING OF PSA TO α₂M**

Different mabs were allowed to react with PSA, followed by incubation with native α₂M. The complexes formed were separated by SDS-PAGE and analyzed by Western blotting with biotin-labeled PSA-C1 as detection antibody (Fig. 5). Compared with the blank (PSA plus α₂M), the binding of PSA to the inhibitor was almost totally blocked by PSA-C2 and PSA-C4, whereas significantly less blocking activity was observed for PSA-C1, PSA-C3, and the irrelevant mab anti-CD91 (compare intensity indexes). Together with the data shown in Table 1, this indicates that the epitope for PSA-C1 may protrude out of the cavity of α₂M. Because we could not exclude the possibility that inhibition of complex formation by PSA-C2 and PSA-C4 is attributable to inhibition of PSA enzyme activity rather than to steric effects, we measured the influence of antibodies on enzyme activity of free PSA (PSA activity index). Whereas PSA-C2 was found to inhibit the enzyme activity (90% inhibition at mab/PSA ratio of 2), the mab PSA-C4 stimulated PSA activity (180% increase at mab/PSA ratio of 2). In contrast, PSA-C3 and PSA-C1 displayed no effect on enzyme activity. Therefore, it is likely that PSA-C4 impairs complex formation between PSA and α₂M through steric effects, as might be at least partially the case with PSA-C3.

![Fig. 4. Detection of PSA-α₂M complexes by different mabs.](image)

**Fig. 4. Detection of PSA-α₂M complexes by different mabs.**

We coated the wells of 96-well plates with different mabs (2 mg/L). After washing, the plates were incubated for 1 h at 37 °C with 200 μL of PSA-α₂M in increasing concentrations. The bound PSA-α₂M was detected by use of HRP-labeled polyclonal anti-α₂M IgG (1:1000).

![Fig. 5. Effect of mabs on PSA activity and binding to α₂M.](image)

**Fig. 5. Effect of mabs on PSA activity and binding to α₂M.**

PSA (5 μg) was allowed to react with equimolar amounts of different mabs for 1 h at 37 °C in PBS. Afterward, a 1/5 molar solution of α₂M (relative to PSA) was added, and the incubation was continued for 1 h. The complexes were then subjected to SDS-PAGE under nonreducing conditions, blotted on nitrocellulose membranes, blocked with 50 g/L nonfat milk in PBS, and analyzed by stepwise addition of biotin-labeled anti-PSA-C1 and HRP-labeled streptavidin, respectively. Lane 1, PSA; lane 2, PSA + α₂M; lane 3, PSA + α₂M + PSA-C3; lane 4, PSA + α₂M + PSA-C2; lane 5, PSA + α₂M + PSA-C4; lane 6, PSA + α₂M + PSA-C1. Semiquantitative analysis was performed by measuring the staining intensity of the α₂M zone in the different lanes, setting lane 1 as 100% (intensity index). The PSA activity index refers to the fraction of PSA activity measured in the presence of PSA-C3 (lane 2), PSA-C2 (lane 3), PSA-C4 (lane 4), PSA-C1 (lane 5), and anti-CD91 (lane 6) compared with PSA activity measured in the absence of the mab (blank = 100%).
To identify mab epitopes in PSA, the mabs were screened with a 7mer phage library. After three rounds of biopanning the selected clones of phages were isolated, their DNA was sequenced, and the amino acid sequences were deduced. The identified heptapeptides and frequencies of clones are presented in Fig. 6A. Several identical clones were selected with high frequency in case of PSA-C2 and PSA-C1, suggesting a high affinity of the mabs to the selected phages and surface-expressed peptides, respectively. For PSA-C4, 10 clones with identical sequences were obtained, whereas no clones with a consensus sequence could be deduced in case of PSA-C3.

The obtained peptide sequences were compared with the PSA sequence (Fig. 6B). None of the peptides showed complete homology with corresponding PSA sequences, indicating the existence of mimotopes. The PSA-C2 sequence (DLVRVIQ) showed similarities with PSA sequences at positions 61–67 (region 1) and at positions 110–116 (region 2). In these sequences, at least three amino acids were identical in position and structure to PSA and were interspaced partially by amino acids of similar physicochemical characteristics, e.g., F vs I in region 1, K vs R and A vs L in region 2. Although a consensus sequence was found in case of PSA-C1 clones with high frequency, this corresponds only in two amino acid positions with PSA (amino acids 9–15), and two amino acids were of similar physicochemical characteristics (W vs Y). Three amino acids in PSA-C4 were found to be identical to positions 69, 71, and 73, spaced by an amino acid with similar physicochemical properties (F vs I). Interestingly, the epitope of PSA-C2 (region 1) overlaps in one position (amino acid 67) with the epitope of PSA-C4. Of 30 clones analyzed by screening with PSA-C3, no consensus sequences could be deduced.

Discussion

Mabs against PSA were raised by immunization of mice with PSA-α₂M complexes for two reasons: (a) to increase the probability of obtaining mabs against PSA epitopes extruding from the inhibitor cavities; and (b) to enhance immunologic response because it is known that α₂M-loaded peptides are effectively endocytosed via the α₂M receptor. This receptor, which is identical to the LDL-related receptor 1 (LRP1 or CD91), is expressed on the surface of antigen-presenting cells and was recently found to promote immune responses by effectively taking up peptide-loaded ligands (11).

α₂M probably plays a major role in the metabolism of PSA and might be important in the development and progression of prostate cancer. There have been reports of very aggressive prostate cancer in patients with a significant α₂M deficiency (12). Conversely, there are reports indicating the correlation of high α₂M concentrations with tumor metastasis (13). Zhang et al. (4) observed that measurement of the ratio of PSA-α₂M to total PSA in serum may improve differentiation between benign prostatic hyperplasia and prostate cancer. Nevertheless, in addition to PSA, α₂M might be a valuable marker for prostate cancer diagnosis.

One of our aims in the present study was to produce mabs that recognize PSA entrapped in α₂M. Usually, small molecules are totally embedded in the two cavities of the inhibitor, as is known to occur for trypsin and α-chymotrypsin (14). On the other hand, there are other proteases that have exposed antigenic sites protruding from the cavities, such as plasmin (15).

In this study we characterized four highly sensitive mabs that were able to detect all three PSA forms, i.e., free PSA, PSA-ACT, and PSA-α₂M. Two separate ELISA protocols were used, which produced different results. When PSA and its complexes were immobilized on the solid phase, only the mabs PSA-C4 and PSA-C1 were able to detect all three PSA forms, whereas the mabs PSA-C3 and PSA-C2 could detect only free PSA. However, when mabs were immobilized on the ELISA plate, all four antibodies were able to detect PSA and its complexes. The differences in recognition are most likely explainable by the fact...
that certain epitopes become nonaccessible through immobilization or that immobilization induces conformational changes leading to the alteration of antigenic areas, a phenomenon that has been described previously (16).

The combination of mab PSA-C1 and PSA-C4-HRP was shown to be highly sensitive to free PSA and PSA-ACCEPT, and it was possible to detect PSA concentrations as low as 10 ng/L in a simple ELISA format. We were able to show that PSA-C4 functions as an activating antibody for PSA-C1, which might be the reason for the high sensitivity of that mab combination. This phenomenon has been also described by Michel et al. (17) who showed that the binding of a mab can change the conformation of PSA, which then can lead to improved epitope accessibility for the second antibody. This is supported by our findings that PSA-C4 was capable of causing an increase in PSA activity, which most probably is attributable to conformational changes.

As far as PSA-α2M complexes are concerned, this is the first report describing mabs capable of detecting PSA caged in the inhibitor. It strongly suggests that parts of the PSA molecule protrude from the inhibitor and are accessible to antibodies. In this respect, PSA resembles plasmin, which can also be detected by antibodies when caged by α2M (15).

To analyze which part of PSA might be exposed from the cavities of the inhibitor, the epitopes of the interacting antibodies were screened by a peptide library. Localizing the binding sites of mabs on their antigens makes them much more useful reagents. Epitopes are known to sometimes be noncontinuous epitopes showing little or no homology with the antigen sequence.

The results of Western blotting experiments indicated that our mabs recognized conformational epitopes of PSA. As expected, we could not find a total alignment of the identified heptapeptides with defined sequences in the PSA molecule. However, it was possible to locate mimotopes that mimic the three-dimensional structure of PSA. In a comprehensive phage display study, Leinonen et al. (18) screened a panel of mabs against PSA with diverse cyclic phage display peptide libraries. In accordance with us, only a few identified peptides showed complete similarity with polypeptide stretches of PSA. Corey et al. (19) located two epitope sites near the PSA amino acid residues 50–64 and 55–69. This region was also recognized by mabs in other studies (20).

We showed that our mabs PSA-C2 and PSA-C4 also recognized amino acid sequences within this region of PSA. In the three-dimensional PSA model, both epitopes, amino acids 61–67 (PSA-C2) and 67–73 (PSA-C4), are very close to each other, making simultaneous binding questionable (Fig. 7). Additionally, PSA-C2 recognizes a second epitope, at amino acid positions 110–116, which is also exposed to the surface. This epitope is more distant from the PSA-C4 epitope. Thus, it could be possible that PSA-C2 and PSA-C4 form a sandwich with PSA as shown in Fig. 2.

![Figure 7](image)

**Fig. 7.** Three-dimensional model of PSA (25) showing the assignment of the different epitopes.

Epitope of PSA-C2, motif 1, region 1 (amino acids 61–67); epitope of PSA-C2, motif 1, region 2 (amino acids 110–116); epitope of PSA-C1, motif 2 (amino acids 9–15); epitope of PSA-C4, motif 3 (amino acids 67–73).

On the other hand, the two epitopes of PSA-C2 are both surface-exposed and are contiguous in the three-dimensional PSA model. It is therefore also reasonable to assume that PSA-C2 recognizes a discontinuous epitope dissected in two stretches. We described such an epitope in human α2M that specifically reacts with a transformation-specific mab (21).

After the ISOBM TD-3 workshop, a panel of antibodies against PSA was categorized into six major groups (22). We would assign the mab PSA-C2 to group 5 antibodies mapped within or closed to the amino acid 58–64 region. Most of the mabs within this group were found to inhibit PSA enzyme activity similar to our mab PSA-C2, as His(37) is part of the catalytic triad.

The epitope of the activity-enhancing mab PSA-C4 could also be projected to a region recognized by group 5 mabs. However, it is known from the literature that many activating mabs bind to the region of group 6 antibodies located at the N-terminal region of PSA (23). We therefore cannot rule out that this mab may recognize an epitope in the N-terminal region that mimics the spatial structure of the displayed peptide.

According to the phage display results, we assigned the mab PSA-C1 to group 6 antibodies because it binds to the N-terminal region of PSA; however, it lacked PSA activity enhancement. Many antibodies within this group were shown to recognize linear epitopes close to the immunodominant sequence (amino acids 3–11) at the N-terminal region of PSA and enhance the enzyme activity of PSA (22). Because in the three-dimensional PSA model a main part of the PSA-C1 epitope is buried and only two amino acids are common with the PSA sequence, PSA-C1 probably recognizes other sequences that mimic the PSA-C1 epitope in conformation (Fig. 7). Alternatively, it could also be that binding of PSA-C4 induces a conformation change in PSA, exposing the hidden
PSA-C1 epitope to the surface. This would explain the high sensitivity for PSA when both mabs are combined (Figs. 1 and 2).

Irrespective of the correct assignment of the PSA-C1 epitope, the observed competition in ELISA experiments clearly demonstrates that the binding regions of PSA-C1 and PSA-C2 might be contiguous.

Regardless of the problems discussed, among the different pairs of antibodies tested the combination of PSA-C4 and PSA-C1 (Table 2) was most sensitive for detecting free PSA and PSA-ACT. The absence of binding interferences in the ELISA indicates that the epitopes are accessible and distant enough from each other to guarantee strong binding.

Because the mabs PSA-C4 and PSA-C1 strongly recognized PSA when bound to α2M, it is suggestive that their epitopes protrude from the α2M cavity. This assumption could be corroborated for PSA-C1 because this mab did not inhibit binding of PSA to α2M, in contrast to other mabs (Fig. 5).

Although we could show that PSA-α2M complexes are detectable by ELISA, further studies need to be done to increase the sensitivity of the test and to establish a setting that allows simultaneous determination of all three forms of PSA. This might be possible by capturing PSA-α2M complexes with a transformation-specific mab directed against α2M-proteinase complexes (21) in conjunction with a labeled anti-PSA mab.

We could further show that recovery of the PSA-α2M complex in PSA-deficient serum is rather high. However, one should consider that there might be more interference when analyzing sera from cancer patients because other forms of PSA might be present. Thus, a high concentration of free PSA or PSA-ACT may disturb the detection of PSA-α2M because of cross-reactivity with the capture antibody. This problem could be circumvented by selective precipitation of the large PSA-α2M complex, e.g., by polyethylene glycol before immunologic testing.

As far as the detection limit of 37.5 µg/L for the PSA-α2M complex is concerned, our value is apparently higher than that of 0.14 µg/L reported for a previously published method using the base extraction procedure (4). However, assuming a 1:1 molar ratio of the complex and a molecular mass of 750 kDa (720 kDa + 30 kDa), this amount corresponds to a PSA concentration of 0.05 nmol/L, which is equivalent to 1.5 µg/L PSA.

Proteases are usually entrapped in the cavities of α2M by covalent bonds. However, a significant part of the protease is physically caged, being freely movable and active (24). This could mean that only a small proportion of PSA in the complex is capable of exposing relevant epitopes. This assumption could partially explain the low sensitivity of our assay in detecting PSA-α2M complexes. Further optimization of our assay will be an important task in the future.

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