Selective Recognition of Enzymatically Active Prostate-specific Antigen (PSA) by anti-PSA Monoclonal Antibodies

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RUNNING TITLE

Recognition of enzymatically active PSA

SUMMARY

Prostate-specific antigen (PSA) is widely used as a serum marker for the diagnosis of prostate cancer. To evaluate two anti-free PSA monoclonal antibodies (mAbs) as potential tools in new generations of more relevant PSA assays, we report here their properties towards the recognition of specific forms of free PSA in seminal fluids, LNCaP supernatants, "non-binding" PSA and sera from cancer patients. PSA from these different origins was immunopurified by the two anti-free PSA mAbs (5D3D11 and 6C8D8) as well as by an anti-total PSA mAb. The composition of the different immunopurified PSA fractions was analyzed and their respective enzymatic activities were determined. In seminal fluid, enzymatically active PSA was equally purified with the three mAbs. In LNCaP supernatants and human sera, 5D3D11 immunopurified active PSA mainly whereas 6C8D8 immunopurified PSA with residual activity. In sera of prostate cancer patients, we identified the presence of a mature inactive PSA form which can be activated into active PSA by use of high saline concentration or capture by an anti-total PSA mAb capable of enhancing PSA activity. According to PSA models built by comparative modelling with the crystal structure of horse prostate kallikrein described previously, we assume that active and activable PSA could correspond to mature intact PSA with open and closed conformations of the kallikrein loop. The specificity of 5D3D11 was restricted to both active and activable PSA whereas 6C8D8 recognized all free PSA including intact PSA, proforms and internally cleaved PSA.

Keywords: prostate cancer, tumor markers, monoclonal antibodies, enzymatic activity, kallikrein.

Prostate-specific antigen (PSA)[1] is a 237-amino acid protein (33 kDa) that belongs to the kallikrein serine-protease family (1) and is encoded by the *hKLK3* gene on the human chromosome 19 (2). The role of PSA is supposed to proteolyse the seminal vesicle-proteins semenogelins I and II, components of the seminal coagulum, immediately after ejaculation (3). However, the PSA may have a number of biological activities in prostate cancer such as the stimulation of cell detachment and thus facilitation of tumor progression or metastasis (4). PSA was also reported as an inhibitor of angiogenesis (5). Thus, it remains an interesting question as to how PSA activity is regulated within the prostate itself, and in the body fluids.

PSA is currently an important serum marker for the diagnosis and follow-up of prostate cancer. In prostate diseases like cancer, benign hyperplasia (BPH) or prostatitis, PSA which is secreted from the prostatic glandular epithelium in the seminal fluid, enters into the circulation, leading to increased PSA concentrations in the serum (6). In the serum, PSA binds to several protease inhibitors, predominantly the α1-antichymotrypsin (ACT) and α2-macroglobulin (A2M), which both inhibit the PSA enzymatic activity (7). Thus, PSA in serum is present both as free PSA and complexed PSA. Moreover, different molecular PSA forms have been characterized either in the seminal fluid (8)-(9) or in sera from patients with cancer or BPH (10)-(11). Indeed, free PSA in serum was shown to contain cleaved (or "nicked") forms lacking enzymatic activity. Furthermore, in a previous study, the use of two-dimensional electrophoresis evidenced that cleaved PSA forms were more abundant in the sera of

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[1] The abbreviations used are: PSA, prostate-specific antigen; BPH, benign prostatic hyperplasia; ACT, α1-antichymotrypsin; A2M, α2-macroglobulin; mAb, monoclonal antibody; NB-ACT PSA, non-binding to ACT PSA; HPK, horse-prostate kallikrein; TPSA, total PSA; FPSA, free PSA; BSA, bovine serum albumin; OD, optical density and MSGK, mouse submandibular gland kallikrein.

BPH patients than in those of prostate cancer patients (12)-(13). Other studies have demonstrated the presence of alternative *hKLK3* transcripts coding for variant proteins of PSA in prostatic tissues (14)-(15)-(16)-(17). These PSA-related forms could be present in biological fluids and could contribute to the free PSA immunoreactive fraction in serum, since they share epitopes with PSA. Their significance for the prostate cancer diagnosis has yet to be demonstrated.

Using the phage display technology, we previously characterized two different conformational epitopes recognized by anti-free PSA monoclonal antibodies (mAbs) (18). On the basis of amino acid similarities between mimotopes and PSA and computer molecular modelling of the PSA three-dimensional structure (19), mAb 6C8D8 recognized the phage displayed peptide RKLRPHWLHFHPVAV, two parts of which presented similarities with two regions distant on the PSA sequence but joined in the three-dimensional structure. MAb 5D3D11 recognized the peptide DTPYWGWLLDEGYD, one part of which was located in a proximal region of the enzymatic site: the residue W205 of the mature form was on the board of the groove containing the active site, whereas the residue S204 was located inside the groove. Both epitopes were localized in the theoretical binding site of ACT. Moreover these mAbs were able to inhibit the enzymatic activity of PSA.

In order to identify these two anti-free PSA mAbs as potential tools for a better diagnosis or prognostic of cancer prostate in new generations of more relevant PSA assays, we further analyzed their properties towards the recognition of specific forms of PSA. In these data we report the biochemical characterization and the enzymatic activity of the different forms of PSA immunopurified from seminal fluids, LNCaP supernatants, “non-binding to ACT” PSA (NB-ACT PSA) and human sera from prostate cancer patients by each of the two anti-free PSA mAbs, as well as an anti-total PSA mAb used as control. The molecular characteristics of the mature PSA forms recognized by 5D3D11 and 6C8D8 are discussed according to the PSA models built by comparative molecular modelling with the crystal structure of horse prostate secreted kallikrein (HPK), the sequence of which presents similarities with human PSA (20).

EXPERIMENTAL PROCEDURES

PSA and antibodies–The human seminal PSA (ref P117-7) and the "non-binding to ACT" PSA (ref P207-3) were purified from human seminal fluid by HPLC. They were supplied by SCIPAC (Sittingbourne, UK).

Anti-total PSA mAbs 11E5C6, 13C9E9 and 8G8F5, anti-free PSA mAbs 5D3D11 and 6C8D8, and anti-proPSA mAb 7D7G7 were produced by bioMérieux (Marcy l'Etoile, France). Conformational epitopes of mAbs 5D3D11 and 6C8D8 in one hand, and 11E5C6 in the other hand were described previously (18), (21). Mab 13C9E9 recognizes a linear epitope on the PSA molecule (E139-P144) (13). Mab 7D7G7 was obtained by immunizing mice with the peptide APLILSR. Mab 8G8F5 had not been described before. Purified mAbs were biotinylated using sulfo-NHS-LC-biotin (Merck) according to Gretch *et al* (22).

Human sera–Human sera from patients with proven clinically prostate cancer were obtained from the Centre Hospitalier Universitaire (CHU) of Liège (Belgium) or from the Société de Secours Minière (Auchel, France). These sera were remnants of samples submitted for routine diagnostic testing. Three pools of sera were prepared and characterized as described on Table I.

Substrates–Chromogenic substrate MeO-Suc-Arg-Pro-Tyr-pNA.HCl (S-2586) was purchased by Chromogenix AB (Mölndal, Sweden). Both fluorometric substrates, morpholinocarbonyl-His-Ser-Ser-Lys-Leu-Gln-7-amino-4-(trifluoromethyl)coumarin (Mu-HSSKLQ-AFC) and morpholinocarbonyl-Lys-Gly-Ile-Ser-Ser-Gln-tyr-7-amino-4-(trifluoromethyl)coumarin (Mu-KGISSQY-AFC) were synthesized by Enzyme System Products (ICN Pharmaceuticals, Aurora, Ohio). These peptides were used previously as PSA substrates (7), (23).

Determination of PSA concentration by VIDAS–Total PSA (TPSA) and free PSA (FPSA) were determined using the VIDAS TPSA immunoassay kit and the VIDAS FPSA immunoassay kit, respectively, from bioMérieux (Marcy l'Etoile, France). The two-step capture/tag tests rely on two mAbs, the detection antibody (mAb 11E5C6) being labeled with alkaline phosphatase.

Culture of LNCaP cells–The LNCaP cell line (American Type Culture Collection ATCC CRL-1740) was derived from human metastatic adenocarcinoma of the prostate (24). The culture was carried out in 125 cm^2 flasks maintained at 37 °C in a humidified atmosphere of 5% CO_2. Cells were first grown in RPMI-1640 medium with phenol red supplemented with 10% fetal bovine serum, 100 U/ml penicillin/streptomycin and 2 mM glutamine until near confluency. Then, the medium was removed and the cells were grown in the same medium containing 1 nM of the synthetic androgen R1881 (NEN-Dupont, Les Ulis, France), which is able to stimulate PSA production. After 5 days, the conditioned medium was collected and another volume of fresh medium containing R1881 was added. This production of PSA was repeated three times (each flask of LNCaP was able to give three conditioned media without decrease of PSA level, data not shown). All the conditioned media were pooled and the TPSA concentration was measured by VIDAS.

Preparation of anti-PSA mAb-bound sepharose–Anti-PSA mAb was coupled to CNBr-activated sepharose according to the procedures provided by the manufacturer (Pharmacia). Briefly, 1 g of dry beads was washed with 1 mM HCl and equilibrated in the coupling buffer (0.1 M $NaHCO_3$ pH 8.3, NaCl 0.5 M). The swollen gel (3 ml) was then incubated overnight at 4 °C with 7 to 9 mg of the anti-PSA mAb/ml of gel. The unreacted sites were blocked with 0.2 M glycine pH 8.0. Unbound mAb was removed with a serie of washes at alternating pH 4.0 and pH 8.0.

Preparation of anti-PSA mAb-coated magnetic beads–10^8 magnetic beads coated with streptavidin (Dynabeads M-280 streptavidin, Dynal, Oslo, Norway) were first washed twice with 500 µl of Bead-Buffer (phosphate buffer 0.1 M pH 7.4, NaCl 0.15 M, Bovine Serum Albumin (BSA) 1 mg/ml) containing Tween 20 (0.5%), and incubated 30 min at room temperature with 500 µl of biotinylated anti-PSA mAb diluted at 20 µg/ml in Bead-Buffer-Tween 0.05%. Then, unreacted sites were blocked by addition of 5 µl of biotin 10 mM. After 30 min of incubation at room temperature, beads were washed five times with 500 µl of Bead-Buffer-Tween 0.5%. Beads are ready to use for immunopurification.

Immunoaffinity purification of LNCaP-supernatant PSA with anti-PSA mAbs bound on sepharose–200 ml of a pool of conditioned media from LNCaP culture containing about 500 µg of PSA was injected to an affinity column of about 1 ml of sepharose-immobilized anti-PSA mAb (about 7 mg mAb/ml of gel), equilibrated in phosphate buffer saline pH 7.2, at 30 ml/h/cm^2. After the sample loading, the effluent was recirculated overnight at room temperature through the column using a peristaltic pump. The day after, the fraction containing the unbound PSA was collected and the bound PSA was eluted with glycine 0.1 M pH 2.8, immediately neutralized to pH 7.2 with Tris 2 M pH 8. TPSA and FPSA concentrations in the different fractions were measured by VIDAS.

Immunoaffinity purification of PSA from pool of sera with anti-PSA mAbs bound on sepharose–1 ml of pool of human sera was added to 50 µl of sepharose-immobilized anti-PSA mAb (9 mg mAb/ml of gel) in an Eppendorf tube and incubated overnight at 4 °C with slow rotation. The tube was then centrifuged and the supernatant was collected. The beads were washed three times with 500 µl of TBS-Tween 0.5% to remove any unbound protein and the PSA-bound to mAb was eluted by incubating the beads with 200 µl of glycine 0.2 M pH 2.2 containing 1 mg/ml of BSA for 5 min at room temperature. The mixture was centrifuged and the eluate was collected and neutralized to

pH 7.2 with 6 µl of Tris 2 M pH 9.6. TPSA and FPSA concentrations in the different fractions were measured by VIDAS.

Immunopurification of PSA from pool of sera with anti-PSA mAbs coated on magnetic beads– 1 ml of pool of human sera was added to 10^8 beads coated with an anti-PSA mAb and incubated overnight at 4 °C with slow rotation. The beads were then washed three times with 1 ml of Bead-Buffer-Tween 0.5% to remove any unbound protein and the PSA-bound to mAb was eluted by incubating the beads with 100 µl of glycine 0.2 M pH 2.2 containing 1 mg/ml of BSA for 5 min at room temperature. The eluate was collected and neutralized to pH 7.2 with 11 µl of Tris 2 M pH 9.6. TPSA and FPSA concentrations in the different fractions were measured by VIDAS.

Two-dimensional electrophoresis and immunodetection of PSA–This method was described previously (12). Briefly, 0.5 µg of PSA was diluted with the rehydratation solution and loaded into a nonlinear IPG strip pH 3-10 (Pharmacia Biotech, Uppsala, Sweden). Then the samples were separated by two-dimensional electrophoresis, transferred onto polyvinylidene fluoride (PVDF) membranes and the PSA was immunodetected with mAb 13C9E9.

N-terminal sequencing of protein–5 to 8 µg of PSA were separated on 12% sodium dodecyl sulfate-polyacrylamide gels as described by Laemmli (25) under reducing conditions, and transferred to PVDF membrane. After staining of proteins with Ponceau S red 0.25% in trichloroacetic acid 3%, the band corresponding to mature PSA (about 30 kDa) was excised from the membrane and directly subjected to Edman degradation on a gas-phase protein sequencer (Procise 492A, Applied Biosystems).

Western-Blot analysis–proPSA transferred on PVDF membrane was immunodetected with anti-proPSA mAb 7D7G7 diluted to a final concentration of 5 µg/ml as described previously (18).

Determination of PSA enzymatic activity with a chromogenic substrate–The PSA enzymatic activity was determined by hydrolysing the substrate MeO-Suc-Arg-Pro-Tyr-pNA.HCl (S-2586) at the final concentration of 5 mM in Tris-HCl 50 mM pH 7.8 NaCl 0.1 M (7). Hydrolysis was measured at 405 nm in a UV recording spectrophotometer (Beckman DU62, Fullerton, CA). All reactions were performed at 37 °C and initiated by the addition of 150 µl of PSA diluted in the reaction buffer. Optical density (OD) is monitored for 30 min. Enzymatic activity is determined by the slope of the kinetic, and specific activity is expressed as OD units/minute/µg of PSA.

Determination of PSA enzymatic activity with a fluorometric substrate–ELISA wells of black plate were coated 2 h at 37 °C with 100 µl of 10 µg/ml streptavidin in 0.05 M carbonate buffer pH 9.6 and blocked for 2 h at 37 °C with TBS (Tris-HCl 50 mM pH 7.5, NaCl 0.15 M) containing 2 mg/ml BSA (TBS-BSA). After 3 washes with TBS-Tween 0.05%, 100 µl of 10 µg/ml biotinylated anti-PSA mAb diluted in TBS-BSA was added. The anti-PSA mAb was either 11E5C6 or 8G8F5. After 2 h of incubation at 37 °C, the wells were washed three times with TBS-Tween 0.05% and 90 to 100 µl of samples containing PSA diluted in TBS-BSA was added and incubated overnight at 4 °C. Following three new washes with TBS-Tween 0.05%, the wells were incubated 15 min at 37 °C with 100 µl of either TBS-BSA or TBS-BSA containing NaCl 1.5 M. Then, the wells were emptied and 100 µl of the PSA substrate (either Mu-HSSKLQ-AFC at 300 µM or Mu-KGISSQY-AFC at 400 µM), diluted in either TBS-BSA or TBS-BSA containing 1.5 M NaCl (among the precedent step) was added. The hydrolysis of the substrate was measured with the Fluoroskan reader (Thermo Life Science, Cergy-Pontoise, France) at 37 °C, every minute for 2 h, using an excitation wavelength of 390 nm and an

emission wavelength of 510 nm. Enzymatic activity is determined by the slope of the kinetic, and is expressed as fluorescence units*1000/minute.

Molecular modelling–The three-dimensional model for the PSA protein was built by using Geno 3D, a comparative molecular modelling of proteins (26). HPK (PDB code: 1gvz-A) and mouse submandibular gland kallikrein (MSGK, PDB code: 1ao5-A) structures were taken as templates for the closed and open conformations, respectively. On the basis of sequence identity after sequence alignment with PSA protein, distance restraints and dihedral angles were calculated on the template structures. These measures were performed for all common atoms revealed by the alignment between the PSA and templates. The CNS 1.1 program (27) was used to generate the model by a distance geometry approach similar to that used in modelling from NMR experiments. Mirror images were eliminated on the basis of energy calculation. Ten models were generated and were superimposed with the ANTHEPROT 3D package (28) by minimizing the root mean square deviation between alpha carbons. The visualization of the molecule was performed with the help of the WebLab ViewerLite (Accelrys, San Diego, California).

RESULTS

Identification the PSA forms present in LNCaP culture supernatants–Human prostate cancer cell line LNCaP expresses both ACT and PSA, and is widely used as a model of prostate cancer *in vitro* as well *in vivo*. Thus, as an approach to characterize the different forms of PSA recognized by the two anti-free PSA mAbs 5D3D11 and 6C8D8, as well as the anti-total PSA mAb 11E5C6, LNCaP culture supernatants were immunopurified with these three anti-PSA mAbs and the different eluates were analyzed by two-dimensional electrophoresis and Western Blot. Using anti-total PSA mAb 13C9E9 as detecting antibody, 5D3D11-immunopurified PSA was detected as a thin band at the apparent molecular weight of about 30-34 kDa corresponding to intact PSA, and as only few spots at lower molecular weights corresponding to cleaved PSA forms (Fig. 1A). By comparison, 6C8D8 immunopurified also intact PSA (larger band at 30-34 kDa) but more cleaved PSA forms (Fig. 1B) and after purification with anti-total PSA mAb 11E5C6, PSA was detected as PSA-ACT complex (spot at the apparent molecular weight of 90 kDa), intact PSA and also numerous cleaved PSA forms (Fig. 1C).

The nature of the PSA detected at 30-34 kDa, and identified as intact PSA in the three eluates was further analyzed by the determination of the N-terminal end of the corresponding extracted proteins. As shown in Table II, intact PSA obtained after immunopurification with 5D3D11 or 11E5C6 was composed of mature PSA only because the sequence of the protein started with the aminoacids IVGG…, whereas PSA immunopurified with 6C8D8 was composed of a mixture of mature PSA as well as two proPSA forms: proPSA(-7) and proPSA(-5). Western-Blot analysis of the same three eluates, as well as the eluate obtained after immunopurification with anti proPSA mAb 7D7G7 as positive control, confirmed the ability of 6C8D8 to recognize proPSA, whereas 5D3D11 and 11E5C6 did not (Fig. 2).

Enzymatic activities of PSA from seminal fluid and LNCaP culture supernatants–The specific enzymatic activity of PSA from seminal fluid was determined in preliminary experiments with chromogenic substrate in solution before and after immunopurification with the three anti-PSA mAbs. The different values were then compared to the values of the PSA activity from LNCaP culture supernatants obtained before and after immunopurification with the same anti-PSA mAbs. As shown in Table III, the specific activity increased after immunopurification of the seminal PSA with the three mAbs. Moreover, no significant difference was observed between the activity of PSA immunopurified with 5D3D11 and the activity of seminal PSA immunopurified with 6C8D8. However, surprisingly, compared to LNCaP PSA activity before immunopurification, the specific activity of 5D3D11-immunopurified-LNCaP PSA almost doubled whereas the specific activity of 6C8D8-immunopurified-LNCaP PSA was residual. These results suggest that 5D3D11 recognized specifically PSA active form whereas 6C8D8 selected in majority inactive PSA composed of proPSA forms but also of other inactive forms including nicked and cleaved PSA. Indeed, in the assay used to quantify the amounts of PSA present in the immunopurification eluates, 11E5C6, which is the detection mAb, does not measure PSA proforms.

Characterization of active and non active mature PSA in LNCaP culture supernatants immunopurified with 5D3D11 and 6C8D8, respectively–We have shown previously that anti-free PSA mAbs 5D3D11 and 6C8D8 inhibited the enzymatic activity of PSA whereas anti-total PSA mAb 11E5C6 had no inhibitory effect on the enzymatic activity of PSA and its capacity to bind ACT (18). However, as shown in Table IV, the binding of another anti-total PSA mAb (8G8F5) to PSA was found to enhance significantly the enzymatic activity of the bound PSA. Moreover, Hsieh and Cooperman (29) have previously found that addition of salts, in particular NaCl, gave rise to important increases of recombinant PSA activity suggesting conformational changes of PSA from a less active to a more active state. Thus, to further analyse the PSA forms selected by 5D3D11 and 6C8D8

respectively, the PSA contained in the two immunopurified eluates was captured by either 11E5C6 (which does not recognize proPSA forms) or 8G8F5 and the measures were carried out either under physiological conditions (NaCl 0.15 M) or at high salt concentration (NaCl 1.5 M). As reported previously (30), the capture of PSA by an PSA-specific mAb allowed to use the highly sensitive fluorometric substrate KGISSQY-AFC in the assays in spite of its lack of specificity (23). Compared to the activities of the eluates in solution (Table III), the capture of PSA by either 11E5C6 or 8G8F5 in the three immunopurified LNCaP-supernatant PSA did not modify the ratios between the respective enzymatic activities (Table V). Then, the use of NaCl 1.5 M or the use of mAb 8G8F5 as capture mAb enhanced significantly the enzymatic activity and the best signal was obtained with NaCl 1.5 M and 8G8F5 as capture antibody. However, even in these optimal conditions, the activity of PSA from LNCaP supernatant immunopurified by 6C8D8 remained residual confirming the enrichment by 6C8D8 of inactive mature and cleaved PSA present in LNCaP culture supernatants but not in seminal fluid, whereas 5D3D11 selected active PSA only.

Enzymatic activity of "non-binding to ACT" PSA–Among the forms of PSA present in seminal fluid, a so called "Non-Binding to ACT" PSA (NB-ACT PSA), because of the inability of this form to complex with ACT, was also obtained after purification of seminal fluid by HPLC (SCIPAC). The N-terminal sequence determination of this PSA form, at the apparent 34 kDa molecular weight, showed that the NB-ACT-PSA was a mixture composed of the mature form (sequence +1: IVGGWEC…) and two truncated forms (+2: VGGWEC… and +3: GGWEC…). As expected, the NB-ACT-PSA did not have any enzymatic activity in regular conditions (NaCl 0.15 M and capture by 11E5C6, Table VI). However, in activating conditions, (NaCl 1.5 M and/or capture by 8G8F5), a significant signal could be detected, suggesting that at least a part of the NB-ACT-PSA was potentially active. The immunopurification of this PSA form by either 5D3D11 or 6C8D8 did not have a significant effect on the enzymatic activity, suggesting that both antibodies selected the same form of PSA which was

present in majority in the NB-ACT-PSA mixture. However, this enzymatic activity remained weak compared to the activity of the same amount of purified seminal PSA.

Characterization of PSA immunopurified from human sera of prostate cancer patients with the immobilized anti-PSA mAbs–Regarding the results obtained with PSA from LNCaP supernatants and NB-ACT PSA, the ability of 5D3D11, 6C8D8 and 11E5C6 to detect active PSA in sera was then tested by measuring the enzymatic activity of different pools of sera from cancer patients with either 11E5C6 or 8G8F5 as capture antibodies. As shown in Table VII, in the absence of activating conditions (NaCl 0.15 M and 11E5C6 as capture antibody), PSA enzymatic activity was not detectable in a pool of sera containing 10.90 ng/ml of free PSA (pool 1) and this activity was residual in the corresponding 6C8D8 immunopurified pool 1. However, PSA contained either in the 5D3D11 immunopurified pool 1 or in the 11E5C6 immunopurified pool 1 was significantly enzymatically active. In activating conditions (NaCl 1.5 M or/and 8G8F5 as capture antibody), enzymatic activity was also found in the pool 1 before immunopurification, as well as after purification with 6C8D8. However, these activities remained weak compared to the activities of PSA immunopurified with either 5D3D11 or 11E5C6.

Thus, as shown in Table VIII, using these conditions of immunopurification, PSA capture and high saline concentration, enzymatically active PSA could be detected in two other pools of sera from patients with prostate cancer containing amounts of FPSA as low as 3.45 ng/ml and 1.31 ng/ml respectively.

Molecular modelling– In order to link our experimental results with structural data, molecular models of the human PSA were built by comparative modeling with HPK (1gvz-A) and MSGK (1ao5-A). The alignment used to perform the model is shown in Fig. 3A. The high identity levels (58,2% with 1gvz-A, 53,5% with 1ao5-A) as well as the absence of gaps strongly support the accuracy of the

models. From these data, one could expect a RMSD deviation in the range 1-1.5Å for alpha carbons with the actual PSA structure, in particular for the catalytic triad (H41, D96 and S189). The models were used to investigate the spatial conservation of essential residues around the crucial S204, reported to be responsible for the change of conformation of the mobile loop 205-210 (loop 215-220 in HPK). This region (203-206) is highly conserved in the three proteins since 3 out of 4 residues are identical and the aromatic feature of W205 is also conserved. Thus, PSA could exist under two conformations (open and closed) equally recognized by 5D3D11. Indeed, as shown in Fig. 3B and C, no obvious difference in the accessibility of the 5D3D11 epitope was observed between the closed and open forms of PSA.

DISCUSSION

The presence of several molecular forms of PSA in human seminal plasma and serum, as well as in the supernatants of LNCaP cultures, has been reported in different studies. In seminal fluid, the majority of PSA is composed of enzymatically active free PSA whereas the remaining PSA consists of either internally cleaved PSA at the C-terminal of R85, K145 and K182 or complexed to protein C inhibitor (31). More than 90% of the PSA from LNCaP cell cultures are free-PSA, but 30% only are active PSA, whereas 40% are zymogen forms and 30% stably inactive PSA (32)-(33). In contrast to seminal fluid and LNCaP cell cultures, in blood, the majority of PSA is complexed with protease inhibitors which are present in serum including ACT and A2M whereas a part remains as a free uncomplexed form despite the presence of a large excess of inhibitors capable of forming complexes with PSA. However the molecular nature of free PSA has not been yet fully explained. Recent studies suggest that free PSA in serum is composed of mature and for some unknown reasons inactive PSA. The presence of proforms of the protein and internal cleavages for example at K145-K146 or K182-S183 are two commonly proposed explanations of free PSA in the circulation. However, Niemela *et al.*, as well as Wu *et al.* have recently detected 1-10% of free active PSA in serum from prostate cancer patients (30), (34).

In our study, the specific enzymatic activities of the different eluates obtained after immunopurification of seminal PSA with 5D3D11, 6C8D8 and 11E5C6 are similar, indicating that the three mAbs are equally able to bind active PSA. Moreover, these results confirm that enzymatically active PSA is the predominant form of PSA in seminal fluid. However, the biochemical characterization of the PSA forms immunopurified from LNCaP culture supernatants by each of the three mAbs and the specific enzymatic activities of the corresponding eluates showed that 5D3D11 has immunopurified enzymatically active PSA only whereas 6C8D8 has immunopurified inactive PSA in majority and, 11E5C6 both active and inactive forms.

As an approach towards the detection of low amounts of active PSA in human sera, the experimental protocol of PSA enzymatic activity determination was optimized by using the sensitive but unspecific fluorometric substrate after capture of PSA by an anti-PSA mAb as already reported (30). Capture of LNCaP-supernatant PSA allowed to measure the activity of PSA contained in eluates immunopurified with 5D3D11, 6C8D8 and 11E5C6 in the absence of activation and, to study the effect of activating conditions. The different ratios between the respective activities of these eluates were similar to those between the activities obtained in solution using the specific chromogenic substrate. This confirmed that the enzymatic activity measured with the fluorometric substrate was specific of PSA activity. In solution, mAb 8G8F5 has been shown to enhance the PSA activity instead of inhibiting it like 6C8D8 or 5D3D11 did. Similarly, the capture of PSA by 8G8F5 coated on solid phase or/and high salt concentration had strong enhancing effects on the PSA activity of the three eluates (4.3, 3 and 7.5 times respectively, compared to the activities with 11E5C6 as capture antibody and salt concentration of 0.15 M NaCl).

Interestingly the use of these activating conditions allowed to show a weak but significant enzymatic activity with the NB-ACT PSA known to be the inactive subfraction of seminal PSA. Moreover, this activated PSA could be equally recognized by both 5D3D11 and 6C8D8. Compared to the 7 times enhancement of the enzymatic activity of active PSA purified from seminal fluid by optimized activating conditions, these activities could not be attributable to an enhancement of residual active PSA present as contaminant in the preparation. Thus NB-ACT PSA, or at least one part of this PSA form, was in fact potentially active and activable.

Recently, Carvalho *et al*. reported the crystal structure at 1.42 Å resolution of HPK purified from seminal plasma (20). HPK shares extensive sequence similarities with PSA including chymotrypsin-like specificity as suggested by the presence of a serine residue at position S1 of the specificity pocket. In contrast to other kallikreins, HPK shows a structurally distinct specificity pocket. Its entrance is blocked by the kallikrein loop. The HPK structure seems to be in an inactivated state and further

processing might be required to allow the binding of substrate molecules. The authors argue that conformational changes may have to occur in order to allow the binding of substrates or inhibitors to HPK, suggesting that the kallikrein loop can exist in two conformations, a closed form in which the access to the active site is restricted and an open form with an accessible active site. Considering the high identity levels and the fact that there is no gaps between the three sequences (PSA, HPK and MSGK), there remains very little doubts about the validity of the PSA three-dimensional models built by comparative molecular modelling. Indeed, most of the conserved residues should occupy the same three-dimensional topology in the three proteins. In particular, the catalytic triad, as well as residues surrounding the crucial S204, are highly conserved spatially. Taken these observations all together, we conclude that the PSA might also exist with open and closed conformations.

Thus, our results suggest that the activable PSA could correspond to PSA in a closed form conformation. Indeed, previous data have reported antigen conformational changes induced by high salt concentration (29) or, in some cases, by monoclonal antibody interaction (35). Moreover, the ability of some anti-PSA mAbs to enhance PSA activity has been already described (36). The experimental conditions used in the assay (1.5 M NaCl and/or capture by 8G8F5) might have changed the conformation of activable PSA into an open form which was enzymatically active.

The data with sera from patients with prostate cancer also support this hypothesis since in activating conditions, enzymatic activity was detected in a pool of sera containing 10.90 ng/ml of free PSA only after prior capture of PSA by anti-PSA mAbs and subsequent washings. This suggests that in sera, the open form of PSA would not be present. Moreover, in solution, activation by high salt concentration would induce a shift from the inactive closed form to active open form which would be immediately inhibited by ACT. The immunopurification of three different pools of sera containing decreasing amounts of free PSA by each of the three mAbs (5D3D11, 6C8D8 and 11E5C6, respectively) gave similar results to those obtained with immunopurification of LNCaP supernatants: the enzymatic activity of 6C8D8 eluate remained weak even in activating conditions compared to the

activity of 5D3D11 eluate and, to a less extent, the activity of 11E5C6 eluate. However, for the first time, enzymatically active PSA could be detected even in sera containing amounts of FPSA as low as 3.45 ng/ml and 1.31 ng/ml respectively.

Regarding the specificities of the three anti-PSA used in this study, the recognition of 5D3D11 would be restricted to both closed and open conformations of mature intact PSA whereas, 6C8D8 would bind all forms of free PSA including proforms of PSA, open and closed forms of intact PSA and internally cleaved PSA. The anti-total PSA mAb 11E5C6 would recognize both open and closed forms of PSA also, as well as nicked PSA and complexed PSA but not, as clearly shown, proforms of PSA. However, compared to the enzymatic activity of 11E5C6, immunopurified PSA from either LNCaP supernatants or human sera, which both contained nicked PSA, the specific activity of 6C8D8 immunopurified PSA in the two eluates for equal amounts of mature PSA was very low suggesting that the affinity of 6C8D8 for the nicked PSA is much higher than the one for the open and closed forms of intact PSA. On the contrary, 11E5C6 would not discriminate intact PSA from nicked PSA. Indeed, in purified seminal PSA as well as in NB-ACT PSA, the presence of nicked PSA was residual thus the enzymatic activities of the three 5D3D11, 6C8D8 and 11E5C6 immunopurified eluates were similar. In sera, the immunopurified intact mature PSA could correspond to the closed form of the PSA kallikrein loop since the open form should be theoretically complexed by ACT.

Previous studies have already reported enzymatically active PSA in sera (30), (34). However, with respect to our results, the experimental protocols were carried in activating conditions with prior capture of PSA by an anti-PSA mAb. In our study, surprisingly, active PSA was detected from human sera after immunopurification with 5D3D11 or 11E5C6 even in the absence of activation. However, the elution of PSA from the immunosorbant after washings in order to eliminate ACT contaminants may have restaured an equilibrium between open and closed forms. This hypothesis is confirmed by the ability of PSA to be still activated with 8G8F5 and high salt concentration.

The epitopes of 5D3D11 and 6C8D8 and 11E5C6 have been previously characterized as conformational epitopes and localized on the PSA molecule (18), (21). Interestingly, one part of the epitope recognized by 5D3D11 was identified at S204, W205 and G206 on the PSA sequence, corresponding to S214, W215 and G216 of the crystal structure of HPK (20). In the HPK structure, loop 215-220 would adopt a conformation that significantly alters the structure of the specificity pocket whereas S214 would be responsible for this distinct conformation bringing the 215-220 loop to the front of the pocket and blocking the access of substrate. According to our PSA models, the 5D3D11 epitope is accessible in both conformational forms of intact PSA.

Free to total PSA has been demonstrated to be higher in patients with BPH compared to those with prostate cancer (37). Moreover, we have previously found that PSA in BPH serum is more extensively nicked than that in prostate cancer serum (13) and, recently, intact PSA has been suggested to be associated with prostate cancer (38)-(39)-(40). Thus, selective assessment of the different molecular forms of PSA to evaluate further the nature of free PSA in serum would improve the diagnostic of prostate cancer. The accuracy of using the different mAbs of this study in combined immunoassays or enzymatic assays for a better discrimination between BPH and prostate cancer is currently under investigation.

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FIGURE LEGENDS

FIG. 1. **Two-dimensional electrophoresis of LNCaP supernatant PSA immunopurified with anti-PSA mAbs.** 0.5 µg of LNCaP supernatant PSA immunopurified by 5D3D11 (*A*), 6C8D8 (*B*) or 11E5C6 (*C*) was detected by mAb 13C9E9 with chemiluminescence technology. *In the bottom*: scale of pI. *In the right*: scale of molecular weight (kDa).

FIG. 2. **Ability of anti-PSA mAbs to immunopurify proPSA.** About 2 µg of LNCaP supernatant PSA immunopurified by mAb 7D7G7 (*lane 1*), 5D3D11 (*lane 2*), 6C8D8 (*lane 3*) or 11E5C6 (*lane 4*) were separated by SDS-PAGE and proPSA was detected by Western-Blot with anti-proPSA mAb 7D7G7. *Lane M*: standard of molecular weight.

FIG. 3. **Molecular modelling of the open and closed PSA conformations**. *A*, multiple sequence alignment of PSA, HPK (1gvz-A) and MSGK (1ao5-A). *B*, representation of the PSA closed conformation based on the HPK structure. *C*, representation of the PSA open conformation based on the MSGK structure. The catalytic triad (common to the three serine-proteases) is composed of H41 (*green*), D96 (*dark blue*) and S189 (*red*). The PSA kallikrein loop (M79-S94) is in *yellow* and the 215-220 loop corresponding to the PSA sequence S204-P209 is in *light blue*. A part of 5D3D11 epitope is included in this loop, whereas 6C8D8 conformational epitope is composed of F57-P59 (*pink*) and K145-Q148 (*purple*).

TABLES

TABLE I

Characterization of three pools of human sera from prostate cancer patients

Sera from patients with proven clinically prostate cancer were pooled. TPSA and FPSA concentrations were measured by VIDAS (bioMérieux, Marcy l'Etoile, France).

Name	Origin	Number of sera included in the pool	TPSA concentration range of individual sera (ng/ml)	TPSA in the pool (ng/ml)	FPSA in the pool (ng/ml)
Pool 1	Société de Secours Minière (Auchel, France)	30	10.20-438.50	71.01	10.90
Pool 2	CHU Liège (Belgium)	35	8.02-62.00	18.49	3.45
Pool 3	CHU Liège (Belgium)	41	4.10-7.93	5.59	1.31

TABLE II

N-terminal sequencing of anti-PSA mAb-immunopurified LNCaP-supernatant PSA

Anti-PSA mAb used for LNCaP-supernatant PSA immunopurification	PSA form detected	N-terminal sequence
5D3D11	Mature PSA	IVGG…
6C8D8	Mature PSA	IVGG…
	ProPSA(-7)	APLILSRIVGG…
	ProPSA(-5)	LILSRIVGG…
11E5C6	Mature PSA	IVGG…

TABLE III

Specific enzymatic activity of PSA immunopurified from seminal fluid and LNCaP supernatant

Enzymatic activity was measured as described in Experimental procedures with chromogenic substrate S-2586 diluted to 5 mM.

	PSA specific enzymatic activity (OD units*1000/minute/µg of PSA) ± standard deviation			
	PSA before immunopurification	PSA immunopurified by anti-PSA mAb		
		5D3D11	6C8D8	11E5C6
Seminal purified PSA	26.4 ± 0.2	38.6 ± 0.1	35.7 ± 0.1	31.0 ± 0.1
LNCaP-supernatant PSA	11.6 ± 1.3	21.6 ± 0.2	1.2 ± 0.4	13.9 ± 3.9

TABLE IV

Inhibition of the enzymatic activity of seminal PSA by anti-PSA mAbs

Seminal purified PSA was preincubated with either a mAb or buffer as control for 30 min at 37 °C (1 mol PSA/1 mol mAb) before being added to 5 mM chromogenic substrate S-2586 at a final concentration of 0.4 µM for both PSA and the mAb.

Anti-PSA mAb	PSA specific activity (OD units*1000/minute/µg of PSA) ± standard deviation
PSA alone	26.4 ± 0.2
Control mAb	26.2 ± 0.5
5D3D11	12.2 ± 0.1
6C8D8	0.2 ± 0.1
11E5C6	27.3 ± 0.4
8G8F5	73.1 ± 1.4

TABLE V

Enzymatic activity of anti-PSA mAb-immunopurified LNCaP-supernatant PSA

Enzymatic activity corresponding to 50 ng/well of immunopurified PSA was measured as described in Experimental procedures with fluorometric substrate Mu-KGISSQY-AFC diluted to 400 µM.

mAb used for LNCaP-supernatant PSA immunopurification	PSA enzymatic activity (fluorescence units*1000/minute) ± standard deviation			
	Capture with mAb 11E5C6		Capture with mAb 8G8F5	
	NaCl 0.15 M	NaCl 1.5 M	NaCl 0.15 M	NaCl 1.5 M
5D3D11	63.8 ± 0.1	193.8 ± 0.1	272.5 ± 0.4	477.3 ± 1.1
6C8D8	3.9 ± 0.0	12.8 ± 0.0	18.0 ± 0.3	34.6 ± 0.0
11E5C6	29.6 ± 0.0	107.2 ± 0.0	136.1 ± 0.2	251.1 ± 0.2

TABLE VI

Enzymatic activity of seminal purified PSA (normal or "non-binding to ACT")

Enzymatic activity corresponding to 20 ng of PSA/well (seminal purified or NB-ACT-PSA) was measured as described in Experimental procedures with fluorometric substrate Mu-HSSKLQ-AFC diluted to 300 μM.

	PSA enzymatic activity (fluorescence units*1000/minute) ± standard deviation			
	Capture with mAb 11E5C6		Capture with mAb 8G8F5	
	NaCl 0.15 M	NaCl 1.5 M	NaCl 0.15 M	NaCl 1.5 M
Seminal purified PSA	18.1 ± 0.1	46.1 ± 0.2	76.4 ± 0.1	130.5 ± 0.2
NB-ACT-PSA	0.4 ± 0.4	7.8 ± 0.1	12.4 ± 0.1	19.8 ± 0.1
5D3D11-immunopurified NB-ACT-PSA	1.7 ± 0.4	8.9 ± 0.5	15.2 ± 0.1	21.5 ± 0.1
6C8D8-immunopurified NB-ACT-PSA	0.3 ± 0.3	9.3 ± 0.1	13.3 ± 0.3	18.9 ± 0.1

TABLE VII

Enzymatic activity of anti-PSA mAb-immunopurified PSA from human sera of prostate cancer patients

PSA from 1 ml of pool 1 of sera was immunopurified (using sepharose beads) with each anti-PSA mAb as described in Experimental procedures. The enzymatic activity was measured with the fluorometric substrate Mu-KGISSQY-AFC diluted to 400 µM and was expressed in fluorescence units*1000/min ± standard deviation. Then, specific activity was calculated for 1 ng of FPSA/well and was expressed in fluorescence units*1000/min/ng of FPSA. N.D. for Not Determined.

		PSA enzymatic activity					
	Added FPSA / well (ng)	NaCl 0.15 M		NaCl 1.5 M			
		Capture with mAb 11E5C6		Capture with mAb 11E5C6		Capture with mAb 8G8F5	
		Measured activity	Specific activity	Measured activity	Specific activity	Measured activity	Specific activity
Pool 1 before immunopurification	0.98	0.0 ± 0.1	0.0	1.0 ± 0.2	1.0	4.9 ± 0.1	5.0
5D3D11-immunopurified pool 1	2.81	9.2 ± 0.1	3.3	29.5 ± 0.0	10.5	62.9 ± 0.0	22.4
6C8D8-immunopurified pool 1	1.98	1.0 ± 0.1	0.5	4.4 ± 0.1	2.2	11.5 ± 0.1	5.8
11E5C6-immunopurified pool 1	N.D.	4.6 ± 0.3	N.D.	16.6 ± 0.7	N.D.	52.8 ± 0.4	N.D.

TABLE VIII

Enzymatic activity of anti-PSA mAb-immunopurified PSA from human sera of prostate cancer patients

PSA from 1 ml of pools 2 or 3 was immunopurified (using magnetic beads) with each anti-PSA mAb as described in Experimental procedures. The enzymatic activity was measured with the fluorometric substrate Mu-KGISSQY-AFC diluted to 400 µM and was expressed in fluorescence units*1000/min ± standard deviation. Then, specific activity was calculated for 1 ng of FPSA/well and was expressed in fluorescence units*1000/min/ng of FPSA.

Pool of human sera	mAb used for immunopurification	Added FPSA / well (ng)	PSA enzymatic activity (capture with mAb 8G8F5, NaCl 1.5 M)	
			Measured activity	Specific activity
pool 2	5D3D11	2.53	19.9 ± 0.2	7.9
	6C8D8	2.86	2.9 ± 0.1	1.0
	11E5C6	3.30	15.7 ± 1.4	4.7
pool 3	5D3D11	0.92	3.3 ± 0.1	3.5
	6C8D8	1.08	1.7 ± 0.1	1.6
	11E5C6	1.28	4.6 ± 0.1	3.6

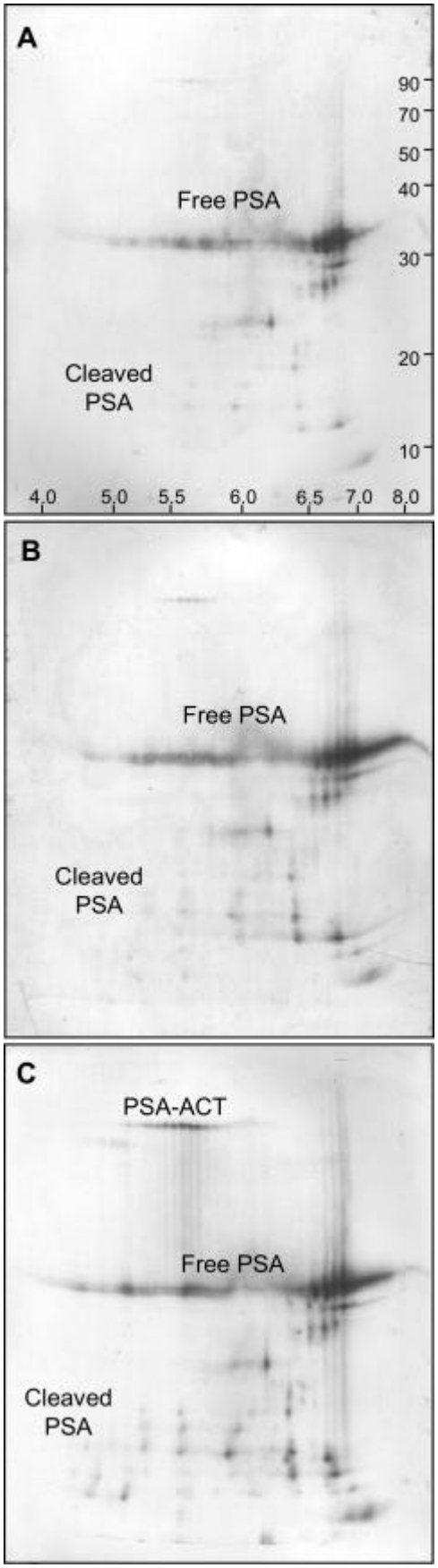

A
90
70
50
40
Free PSA
30
20
Cleaved
PSA
10
4,0
5,0
5,5
6,0
6,5
7,0
8,0
B
Free PSA
Cleaved
PSA
C
PSA-ACT
Free PSA
Cleaved
PSA

FIGURE 1

FIGURE 2

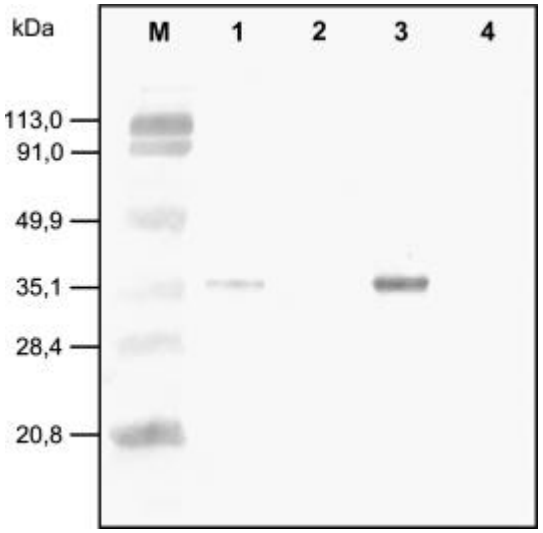
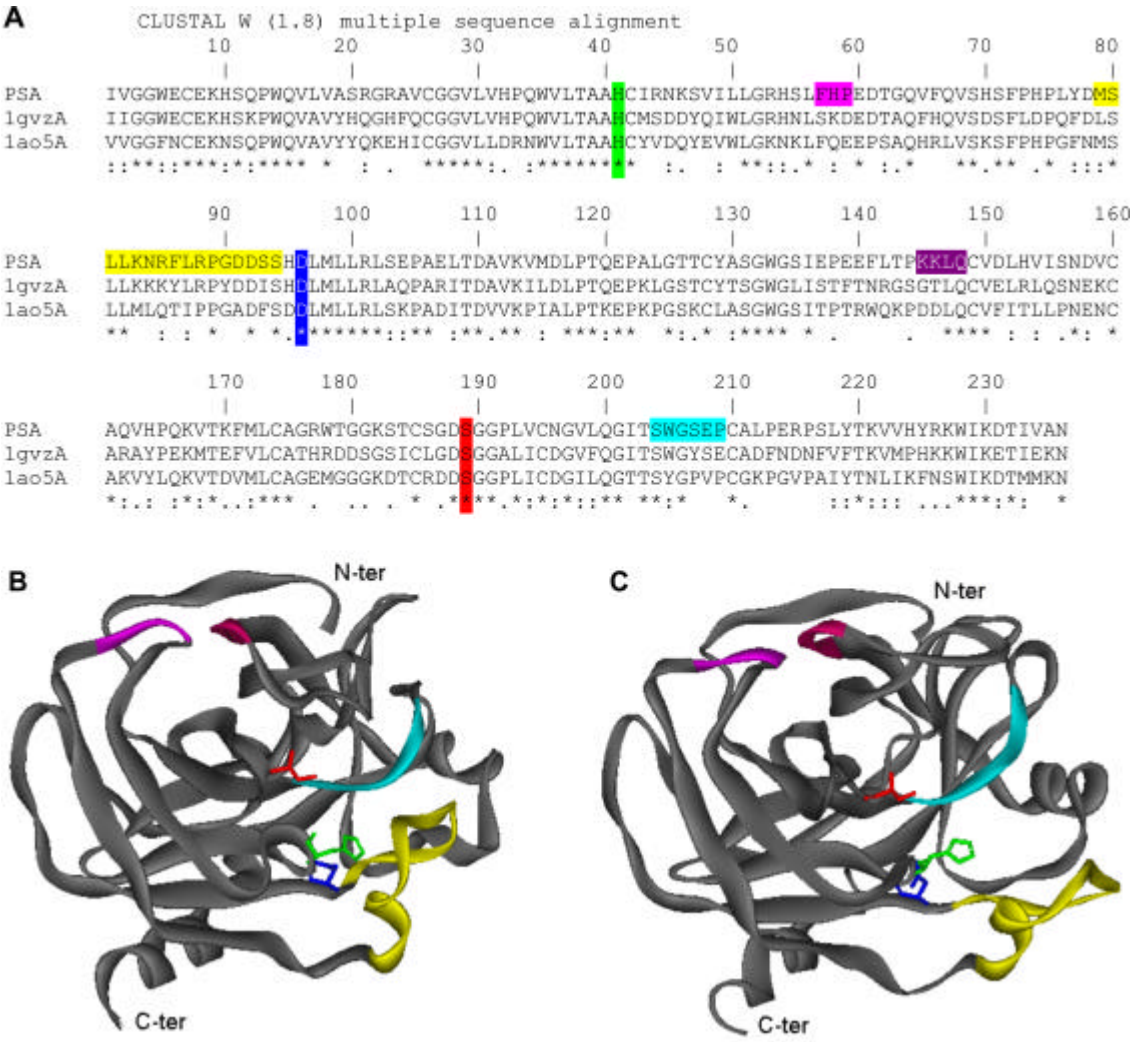


FIGURE 3


A
CLUSTAL W (1.8) multiple sequence alignment
PSA IVGGWECEKHSQPWQVLVASRGRAVCGGVLVHPQWVLTAAHCIRNKSVILLGRHSLFHPEDTGQVFQVSHSFPHPLYDMS
1gvzA IIGGWECEKHSKPWQVAVYHQGHFQCGGVLVHPQWVLTAAHCMSDDYQIWLGRHNLSKDEDTAQFHQVSDSFLDPQFDLS
1ao5A VVGGFNCEKNSQPWQVAVYYQKEHICGGVLLDRNWVLTAAHCYVDQYEVWLGKNKLFQEEPSAQHRLVSKSFPHPGFNMS
PSA LLKNRFLRPGDDSSHDLMLLRLSEPAELTDAVKVMDLPTQEPALGTTCYASGWGSIEPEEFLTPKKLQCVDLHVISNDVC
1gvzA LLKKKYLRPYDDISHDLMLLRLAQPARITDAVKILDLPTQEPKLGSTCYTSGWGLISTFTNRGSGTLQCVELRLQSNEKC
1ao5A LLMLQTIPPGADFSDDLMLLRLSKPADITDVVKPIALPTKEPKPGSKCLASGWGSITPTRWQKPDDLQCVFITLLPNENC
PSA AQVHPQKVTKFMLCAGRWTGGKSTCSGDSGGPLVCNGVLQGITSWGSEPCALPERPSLYTKVVHYRKWIKDTIVAN
1gvzA ARAYPEKMTEFVLCATHRDDSGSICLGDSGGALICDGVFQGITSWGYSECADFNDNFVFTKVMPHKKWIKETIEKN
1ao5A AKVYLQKVTDVMLCAGEMGGGKDTCRDDSGGPLICDGILQGTTSYGPVPCGKPGVPAIYTNLIKFNSWIKDTMMKN
B
N-ter
C-ter
C
N-ter
C-ter