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Recombinant PSP94 (Prostate Secretory Protein of 94 Amino Acids) Demonstrates Similar Linear Epitope Structure as Natural PSP94 Protein

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Abstract PSP94 has the potential to be a useful diagnostic marker and therapeutic agent in prostate cancer. Recently, different immunoassay systems for quantitative analysis of PSP94 in clinical samples have been developed, but the epitope structure of PSP94 protein has not been elucidated. In this study, we report an *Escherichia coli* expression system for recombinant GST–PSP94 fusion protein. GST–PSP94 contains antigenic determinants similar to natural PSP94 protein (determined both by Western blotting experiments and by ELISA) and can be used to study the structure of natural PSP94 antigen. Since GST–PSP94 was expressed in *E. coli* and purification involved a denaturing process, we propose that the epitope structure of PSP94 is linear and largely dependent on the primary amino acid sequence, rather than conformational structure. This hypothesis was supported by reciprocal competition in ELISA among natural, GST–PSP94 fusion protein, and purified recombinant PSP94 protein. The results demonstrate that the various forms of PSP94 can compete with each other in binding to rabbit PSP94 polyclonal antibody, although the natural PSP94 has a slightly higher affinity. When natural and recombinant PSP94 protein were denatured in vitro with urea and alkali, no effect on the binding to antibody was found. The epitope activity of natural PSP94 was also shown to be resistant to the treatment of detergent and reducing agent. The location of one of the linear epitopes recognized by the PSP94 antibody was determined to be in the N-terminus by using two synthetic peptides representing N- and C-terminal sequences. Competitive ELISA between the N-terminal peptide and PSP94 protein indicate that both natural and GST–PSP94 have similar immunoactive N-termini.

Key words: recombinant GST–PSP94, linear epitope, antigen binding, peptide mapping, ELISA, competitive ELISA, immunoassay

Human PSP94, also designated β-microseminoprotein (β-MSP), is one of the three predominant proteins—PSP94, prostatic acid phosphatase (PAP), and prostate-specific antigen (PSA)—secreted by the prostate gland [Carter et al., 1982; Tremblay et al., 1987; Dube et al., 1987; Abrahamsson et al., 1988; for review, see Hara et al., 1989]. The primary structure of PSP94 protein is known from amino acid sequence determination of the purified protein from seminal plasma [Seidah et al., 1984; Akiyama et al., 1985], cDNA sequencing [Mbikay et al., 1987; Ulvsback et al., 1989], and genomic sequencing [Green et al., 1990; Nolet et al., 1991; Ulvsback et al., 1991; Ochiai et al., 1995]. PSP94 is a single polypeptide chain without a carbohydrate moiety, highly charged and cysteine-rich, and devoid of alanine. PSP94 has been demonstrated to be one of the sperm-coating antigens [Johansson et al., 1984; Akiyama et al., 1985; Hara et al., 1989]. Recently, alternative splicing of PSP94 mRNA in prostate tumor tissues [Xuan et al., 1995a] and the promoter region responsible for cell-specific expression [Ochiai et al., 1995], have been identified.

PSP94 was reported to inhibit in vivo at an endocrine level the secretion of β-follicle-stimulating hormone (β-FSH) from the pituitary gland and for this reason, it has also been called β-inhibin and prostatic inhibin peptide (PIP) [Sheth et al., 1988]. At the paracrine or autocrine level, PSP94, synthesized directly by prostate tissue may inhibit prostatic tissue growth [Sheth et al., 1988; Lokeshwar et al., 1993; Mundel et al.,

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1993; Garde et al., 1993a]. Studies have indicated apoptosis in rat prostate exposed to increased levels of exogenous PSP94 [Mundel et al., 1993]. Several research reports [Sheth et al., 1988; Lokeshwar et al., 1993; Mundel et al., 1993] showed that PSP94 may represent a novel and potentially nontoxic form of therapy for hormone-independent prostate cancer.

The presence of PSP94 has been analysed by two-dimensional gel electrophoresis [Carter et al., 1982] radioimmunoassays (RIA) [Vaze et al., 1979; Tremblay et al., 1987; Huang et al., 1992; Huang et al., 1993], immunohistochemistry (IHC) [Doctor et al., 1986; Dube et al., 1987; Abrahamsson et al., 1988], in situ hybridization [Mbikay et al., 1987; Brar et al., 1988], and enzyme immunoassays [Teni et al., 1989]. By immunoassay of protein levels in blood samples from both benign prostatic hyperplasia (BPH) and neoplastic tissues, PSP94 was reported, as compared with two other prostate tumor markers PAP and PSA, to be a potential biomarker for monitoring the course of prostate carcinoma (PCa) [Sheth et al., 1987; Teni et al., 1988; Garde et al., 1993b; Huang et al., 1993]. They are quite different, however, with respect to their responses to endocrine treatment of PCa patients. PSA and PAP are primarily dependent on androgens, while PSP94 has been reported to be androgen independent [Garde et al., 1993a,b].

As an antigen, PSP94 was reported to bind with two kinds of antibodies: polyclonal or monoclonal antibodies against natural PSP94 (nPSP94) purified from seminal plasma; and monoclonal antibody (*CD16leu11b*) against IgG receptor FcγRIII [Liang et al., 1991, 1992]. Both polyclonal and monoclonal antibodies have been developed by several research laboratories [Vaze et al., 1979; Wright, Jr. et al., 1990], and different RIA or two-site immunoradiometric methods were also established [Vaze et al., 1979; Huang et al., 1992]. However, the epitope structure of PSP94 has not been determined. As an immunoglobulin binding factor (IgBF), PSP94 can bind with the Fc fragment of IgG and acts as an IgG receptor [Liang et al., 1991, 1992; Maeda et al., 1993]. Thus, PSP94 may play a role as an immunosuppressive factor in seminal plasma and influence fertilization, implantation, and pregnancy by preventing sensitization of female reproductive cells [for review, see Fridman, 1991]. The function of specific regions and structures of PSP94 in this binding, however, is unknown.

In this article we report the establishment of an *Escherichia coli* system for the expression of recombinant GST (glutathione S-transferase)-PSP94. Using recombinant GST–PSP94 fused protein, we have found that most of the epitopes of both natural and recombinant PSP94 have a linear structure. We propose that GST–PSP94 protein is a useful model for further study of PSP94 function.

MATERIALS AND METHODS

Peptides Synthesized

C-terminal 28-amino acid peptide ($C28^{67-94}$), I^{67}FKKEDCKYI VVEKKDPKKT CSVSEWII^{94}, Cys-ACM(acetamidomethyl)^{73,87}, N-terminal 30-amino acid peptide ($N_{30}{}^{1-30}$) S^{1}CYFIPNEGV PGDSTRKCMD LKGNKHPINS^{30}, Cys-ACM^{2,18}, were synthesized by an automatic peptide synthesizer (model 9050, Milligen). The peptides were dialyzed in Spectra/Por membranes (MWCO: 1,000, Spectrum, Houston, TX) for 4 days. Peptide concentration was estimated by measuring $OD_{280\,nm}$ and using originally synthesized peptide (5 mg/ml) as control.

Construction of GST–PSP94 Fusion Plasmid in *E. coli* Expression Vector PGEX2T

A PSP94 cDNA clone was isolated and sequenced as reported previously [Xuan et al., 1995a]. Two expression vectors directing production of recombinant proteins fused to GST or polyhistidine systems have been assessed. Only the GST system was successful. The mature PSP94 coding region was cloned into the *E. coli* expression vector pGEX2T (Pharmacia, Montreal, Que) by PCR. Two primers located near the N-terminus of the excision site of PSP94 precursor protein (PR1) and the stop codon (PR2), were synthesized (Procyon, Ont). The N-terminal primer PR1 (GGGTTGGATC CTGCTATTTC ATACCTAAT) was synthesized (Fig. 1A) to maintain the same reading frame as the carrier protein GST, and contain a cloning site of restriction enzyme *Bam*HI. PR2 consists of the 3′ end of PSP cDNA (GGGAAGAATT CTTAGATTAT CCATTCACT) and contains an *Eco*RI restriction site. PCR was performed under previously reported conditions [Xuan et al., 1995a] using 10 ng of the PSP94 cDNA template. PCR product was purified by the Glassmatrix method (Gibco/BRL, Burglinton, ON), digested with *Bam*HI and *Eco*RI enzyme, and ligated into a pBS plasmid (Stratagene, CA), previously linear-

ized by the same restriction enzymes. A recombinant clone was characterized by physical mapping and DNA sequencing. All the related recombinant DNA techniques (restriction, ligation, transformation, screening, and DNA sequencing analysis) were performed using standard protocols [Sambrook et al., 1989] or as we have previously reported [Xuan et al., 1995a,c]. The *E. coli* expression vector pTricHis C (Xpress system, Invitrogen, San Diego, CA) was used to insert a full-length PSP94 cDNA sequence bounded by *Bam*HI and *Eco*RI sites, and maintained in the same reading frame.

Expression and Purification of Recombinant GST–PSP94

Recombinant GST–PSP94 was expressed in the *E. coli* strain BL21 (Novagen, Madison, WI), which lacks the *lon* protease and the *omp*T outer membrane protease. *E. coli* bacterial culture, the induction of recombinant protein by 0.5 mM IPTG (isopropylthio-β-D-galactoside) and affinity purification of the GST–PSP94 fusion protein using Glutathione Sepharose 4B (Pharmacia, Montreal, Que) were performed as reported previously [Xuan et al., 1994, 1995b]. Large scale (1.5-L LB media) preparation of the fusion protein was performed in a Biostat M fermentator (B. Braun), at 32–34°C with agitation at 800 rpm. Recombinant GST–PSP94 expression was induced at OD_{600} of 5.0–5.5. Large inclusion bodies were typically visible under the microscope 2 hours post-induction. The cell pellet was collected within 3 h of IPTG treatment and the GST-PSP protein in inclusion bodies part was purified according to the protocol as previously reported [Frorath et al., 1992; Xuan et al., 1994]. In brief, a denaturing solution (8 M urea, 0.1 M glycine pH 9.0) was employed to dissolve all of the precipitated proteins in the inclusion bodies and was immediately desalted by passing through a Sephadex G50 column in 0.1 M glycine pH 9.0 buffer. Renaturation of the desalted protein fractions was obtained in the same buffer at 4°C overnight. Thrombin digestion was performed at a dosage of 10 U/mg protein as previously reported [Xuan et al., 1994]. After digestion, recombinant PSP94 (rPSP94) was further purified by passing through GST affinity column. 10% Triton X-100 was added to the thrombin digested GST–PSP94 mixture at a final concentration of 1%, about 10% volume of Glutathione Sepharose was added and incubated for 1 h. The protein eluate was desalted in Centricon-3 centrifugal concentrators (Amicon, Beverly, MA). Protein concentration was determined by the Bradford method (BioRad protein assay kit, BioRad, Mississauga, ON).

Purification of Natural PSP94 (nPSP94) Protein

Human seminal plasma was collected from clinic laboratory and diluted three times with DMEM media and centrifuged to remove sperm. The supernatant was precipitated by addition of ammonium sulphate and purified by ion-exchange chromatography (M. Baijal et al., manuscript in preparation).

Western Blotting

Rabbit polyclonal antibody against nPSP94 (a gift from Dr. Seema Garde) purified from seminal plasma was obtained by active immunization [Vaze et al., 1979; Vanage et al., 1992], in which the purified PSP94 was mixed with Freund's adjuvant without denaturation. Chemiluminescence method was followed using an ECL Western blotting kit (Amersham, Oakville, Ont) according to the protocol provided by the manufacturer. Ten to 50 ng of protein samples were boiled and loaded onto 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and electrotransferred to nitrocellulose membrane (Hybond-ECL membrane, Amersham). Primary antibody and the secondary antibody, horseradish peroxidase (HRP)-conjugated swine antiserum against rabbit IgG (Dimension Laboratories, Mississauga, ON), were diluted 5,000 and 1,000 times separately.

Direct ELISA

Protein samples in carbonate coating buffer (1.4 mM Na_2CO_3, 7 mM $NaHCO_3$ pH 9.2) were coated in triplicate onto 96-well Immunoplates (Nunc, Gibco/BRL, Mississauga, ON) at 4°C overnight. The coated plate was washed three times in phosphate-buffered saline (PBS)/T buffer (80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, 0.05%/Tween 20), and blocked in 1.5% bovine serum albumin (BSA, RIA grade, Sigma, St. Louis, MO) in PBS at 37°C for 1 h. The wells were washed briefly and incubated at room temperature with antibody diluted (1: 10,000) in 1.5% BSA/PBS/T for 1 h at room temperature. This immunoreaction was terminated and washed three times with PBS/T. A secondary antibody, horseradish peroxidase (HRP)-conjugated swine antiserum antirabbit

IgG, was diluted in PBS, added to each well and incubated at room temperature for 1 h. The plate was washed three times with PBS. Color reaction was in 0.4 mg/ml OPD (*o*-phenylene diamine dihydrochloride; Sigma, St. Louis, MO) 0.05% H_2O_2 in developing buffer (35 mM citric acid, 67 mM Na_2HPO_4, pH 5.0), and incubated at room temperature for 20 min, stopped by the addition of an equal volume of 1 N H_2SO_4. Results were measured by OD_{492nm} using a Titrek Multiscan Plus (EFLAB). Graphs were generated and statistical analyses were performed using SigmaPlot and SigmaStat software (Jandel Scientific Co.).

ELISA of the in vitro denatured protein. About 1–2 μg of protein samples was lyophilized and redissolved in 10 μl of the following denaturing solutions: 8 M urea 0.1 M glycine pH 9.0, 1% SDS 0.01% 2ME (β-mercaptoethanol); 10 μl double-distilled water was used for the control and boiling test. Denaturing conditions was maintained for 3 min at room temperature (except for boiling test), at least 1,000 times of enzyme-linked immunosorbant assay (ELISA) coating buffer was added immediately to all the samples to a final concentration at 50 or 100 ng/100 μl. Coating and renaturing were at 4°C overnight, then proceeded as for direct ELISA.

Competitive ELISA

Coating and blocking of the antigen sample wells (solid phase) were performed as for the direct ELISA. To assess the ability of antigen in solution phase in inhibiting antibody binding to solid phase antigen, PSP94 antiserum was diluted 1:10,000 in 1.5% BSA/PBS/T and mixed with the competitor proteins, either peptides or three sources of PSP94 antigens (nPSP94, GST-PSP94, and rPSP94) as solution phase at various concentrations. Antibody-competitor mixture was added immediately to the sample wells in triplicate and incubated at room temperature for 2 h with shaking. The competition reaction was stopped by washing three times with PBS/T. HRP conjugated secondary antibody binding and colour development with OPD were performed as for the direct ELISA described above.

Competitive ELISA using GST–PSP94 protein to generate standard curves for use in quantitating PSP94 in clinical samples was performed in a similar fashion as for competitive ELISA. Briefly, 50 ng (~5 pmol) of PSP94 protein was coated as an antigen in solid phase on plastic well overnight at 4°C and blocked for at least 1 h. PSP94 antiserum was diluted (1:25–40,000) and preincubated with standard PSP94 protein (competitor antigen in solution phase) at dosages ranging from 0.19 (~0.019 pmol) to 100 (~10 pmol) ng/ml for two hours. The mixture was transferred to the wells coated with antigen to determine the remaining unbound excess antibody. Antibody binding reaction on plastic wells was maintained for another 2 h and terminated by washing. Secondary antibody and color development were performed as described for the direct ELISA. Standard curves were plotted as relative absorbency (B/B0) against competitor standard protein (ng/ml). Relative absorbency (B/B0) was calculated as follows: B = OD_{492} of the sample −NSB. Nonspecific binding (NSB) was determined by testing OD_{492nm} with excess standard (1 μg) in the competition reaction to entirely block antibody binding, i.e., under maximum competition and minimum antibody binding to the plate, B0 = OD_{492nm} of the maximum antibody binding (no competition) − NSB.

RESULTS

Characterization of the Ability of Recombinant GST-PSP94 Binding With Polyclonal Antibody Against Natural PSP94

In order to manipulate cDNA and protein sequence of PSP94, we first generated an GST expression system in *E. coli*. As shown in Figure 1A, the N-terminus of the recombinant PSP94 protein was designed to have only one extra amino acid (Gly) in the N-terminus of mature PSP94. This Gly is part of both the *Bam*HI cloning site and thrombin cleavage site. The time course of the expression of GST–PSP94 fusion protein in *E. coli* of the IPTG induction showed the production of a unique 35 kDa protein (data not shown). The recombinant PSP94 protein was found in both cytosol and inclusion body fractions in *E. coli* lysate, but the majority (more than 95% of total recombinant protein) was precipitated in the cell fraction of inclusion bodies. As shown in Figure 1B, the majority of the precipitated protein trapped in the inclusion bodies inside the *E. coli* cells was GST–PSP94 (lane 1). After GST–Glutathione Sepharose affinity column purification, GST–PSP94 protein purified from the cytosol (lane 4) is less pure than that in the inclusion bodies (lane 2); i.e., there is a minor truncated form of GST–PSP, as found with other recombinant GST proteins [Xuan et al., 1994, 1995; Bautista et al., 1994].

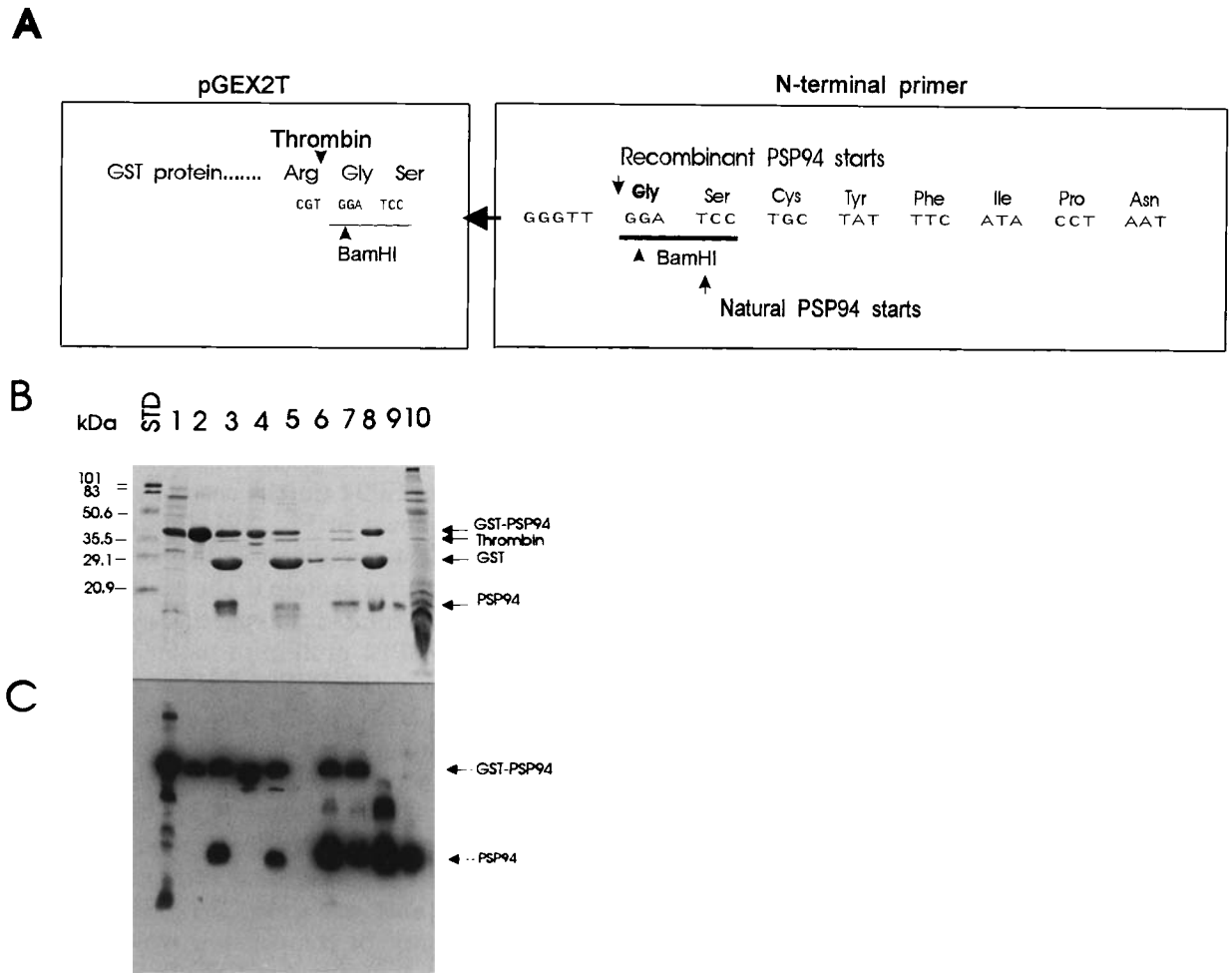


Fig. 1. Expression of recombinant GST-PSP94 fusion protein in *E. coli* cells. **A:** PCR cloning of the mature PSP94 cDNA sequence in an *E. coli* expression vector pGEX-2T. Upstream primer (29 mer) is shown in the left box. The *Bam*HI recognition sequence in both vector (right box) and N-terminal primer is underlined; *arrows*, digestion site. *Arrow* (top), thrombin cleavage site. The extra glycine residue in GST–PSP94 is shown in bold. Two boxes also show that the *Bam*HI site in vector and N-terminus sequence of rPSP94 is in the same reading frame. **B:** 15% SDS–PAGE analysis of the expression of GST–PSP94 protein in both cytosol (*lanes 4,5*) and inclusion body (*lanes 1–3*) fractions of the bacteria cell. *Lane 1*, inclusion body protein dissolved in 8 M urea, 0.1 M glycine pH 9.0. Total cellular inclusion body fraction was obtained as a precipitate in the crude cell lysate by lysozyme and 1% Triton X-100 treatment of bacteria cell pellet [Xuan et al., 1994; Frorath et al., 1992]; *lane 2*, purified GST–PSP94 protein from inclusion body (sample of *lane 1*) by Glutathione Sepharose 4B affinity column; *lane 3*, sample of lane 2 digested by thrombin; *lane 4*, affinity column-purified GST–PSP94 from cytosol fraction of bacteria cell; *lane 5*, sample of lane 4 digested by thrombin; *lane 6*, GST protein digested by thrombin; *lane 7*, crude preparation of rPSP94 protein (sample of lane 3 passing through affinity column purification); *lane 8*, eluate from affinity column purification of the sample in lane 3 absorbed in the column; *lane 9*, purified nPSP94 from seminal plasma; *lane 10*, supernatant from seminal plasma. **C:** Western blotting analysis of 15% SDS–PAGE of B. About 5% of all the 10 samples in B were loaded and blotted (see Materials and Methods).

Both fractions of GST–PSP94 fusion proteins are resistant to the complete digestion by thrombin as shown in lanes 3 and 5, since increasing the dosage of thrombin enzyme to an excess amount still only resulted in partial digestion (data not shown). The partial digestion of GST–PSP94 by thrombin is also likely due to poor solubility of GST–PSP94 protein. Reapplication of thrombin-digested GST–PSP94 protein to the affinity column for purification did improve purity but with significant loss of recombinant PSP94 (rPSP94) protein (cf. lanes 7 and 8).

To verify the identity of the recombinant PSP94 protein, blots similar to those shown in Figure 1B were tested with rabbit polyclonal antibody against natural PSP94. GST–PSP94 reacted positively with PSP94-specific antibody (Fig. 1C), as did the two positive controls of

nPSP94, either purified (lane 9) or in the seminal plasma (lane 10). In all cases, the carrier protein GST (27 kDa) and thrombin enzyme were negative to PSP94 antibody, even when overloaded in these lanes (lanes 3, 5, 8). Therefore GST–PSP94 fusion protein was used as a experimental model to study the structure of the PSP94 antigen.

Test of Recombinant GST–PSP94 as a Standard Coating Antigen and Competitor Protein in Immunoassay

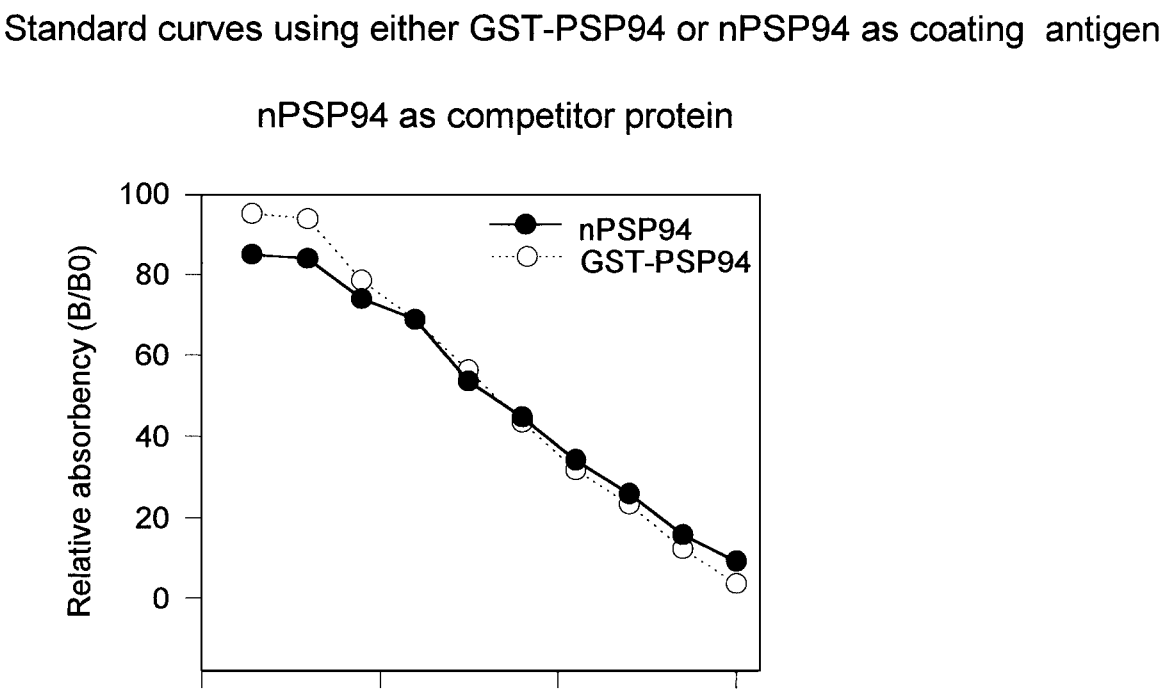
We tested whether GST–PSP94 could be detected qualitatively and quantitatively by PSP94-specific antibody in order to assess the use of recombinant PSP94 to replace nPSP94 as a standard protein in immunoassays of the levels of PSP94 in clinical samples. Two sets of immunoassays for obtaining standard curves were conducted. First, the ability of GST–PSP94 protein to replace nPSP94 as a coating antigen (solid phase) was determined. In the assay (Fig. 2A), either nPSP94 (solid line) or GST–PSP94 (dotted line) was coated and bound to plastic well (~5 pmol) as a solid-phase antigen and nPSP94 was in the solution phase as the competitor. The binding response (relative absorbency) tested for either nPSP94 or GST–PSP94 was measuring the excess antibody remained in the solution phase after competition with various amount of standard nPSP94 (0.1–100 ng, i.e., ~0.01–10 pmol). As shown in Figure 2A, the two standard curves generated for the two kinds of coating antigens in solid phase were similar, with close to the same slopes and they are overlapping in most of the dose range tested. Thus, recombinant GST–PSP94 can be used as appropriate solid phase for a coating antigen in an immunoassay to measure natural PSP94 in clinical samples. Next, the capacity of GST–PSP94 to be used as antigens in both solid and solution phases for quantitation was assessed. In this test (Fig. 2B), only GST–PSP94 was used as coating antigen (solid phase), solution phase antigen nPSP94 (solid line) was compared to solution phase GST-PSP94 (dotted line) with respect to their ability to compete for binding with solution phase PSP94 antiserum. As shown in Figure 2B, the two standard curves did not overlap and GST–PSP94 gave a steeper slope than did nPSP94.

Reciprocal Competitive ELISA Between Natural and Recombinant GST–PSP94

To quantify further the differences of the epitope structure and affinity of both nPSP94 and GST–PSP94 proteins binding with the polyclonal antibody, reciprocal, competitive ELISA experiments between these two proteins were performed. In the assays, increasing amounts of nPSP94 (Fig. 3A) or GST–PSP94 (Fig. 3B) were coated as solid-phase antigen. The capacity of solution phase anti-PSP94 antibody to bind with the solid phase was tested in the presence of competitor solution phase antigen GST–PSP94 (in the case of solid-phase nPSP94, shown in Fig. 3A) or nPSP94 (in the case of solid-phase GST–PSP, shown in Fig. 3B). Control experiments were tested as direct ELISA without adding any competitor protein in the solution phase. The reciprocal competition partners were 50 ng (~5 pmol) PSP94 protein in molar equivalent (per well), i.e., GST–PSP94:PSP94 (37,813 Da: 10,770 Da) ~3.5:1, mixed with a limiting amount of PSP94 antiserum (1:10,000 dilution). The results shown in Figure 3 represent the competitive binding of both sources of PSP94 proteins with the polyclonal antibody. Comparing Figure 3A and B, nPSP94 showed stronger binding with PSP94 antibody than GST-PSP94 in the high-dose range of competition which started from 10 ng (~0.25 pmol) of GST-PSP94 or 5 ng (~0.5 pmol) of nPSP94, till saturated binding level at about 100 ng (~3–10 pmol) for both proteins. In the lower dose range, <10 ng (~0.25 pmol) for GST–PSP94 or 3 ng (~0.3 pmol) of PSP94, both proteins have similar extent of inhibition to the partner protein. This result was summarized in Figure 3C, in which competition inhibition was statistically analysed by the proportion of the decreased OD_{492nm} mean value with that of the control. The results of this experiment indicate that both proteins have the similar domain of epitopes, but in GST–PSP94 this epitope was suppressed within a specific range.

To determine whether the differences in affinity between GST–PSP94 and nPSP94 with antibody was due to the presence of GST carrier protein, recombinant PSP94 protein (95 amino acids residues, MW 10,845 Da) was released from GST protein by thrombin digestion and purified by passing through the substrate (glutathione) affinity column. As shown in Figure 1B, the crude preparation of rPSP94 was relatively pure after passing through the second affinity

A



B

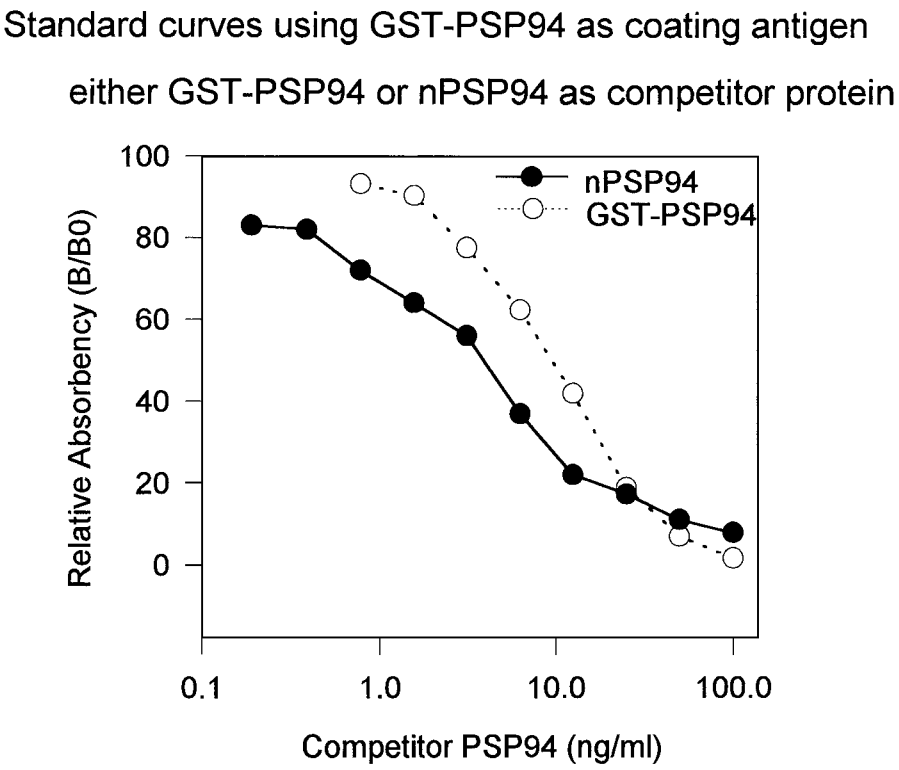
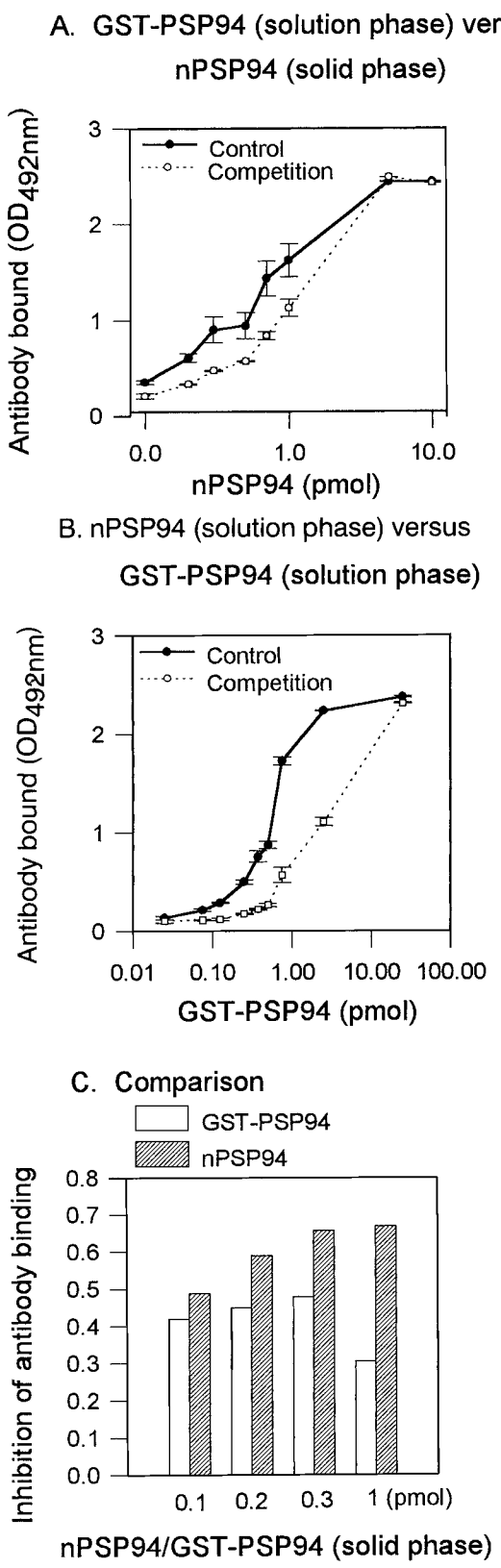


Fig. 2. Standard curves in immunoassays using nPSP94 and recombinant GST-PSP94 as standard or coating antigen. **A:** Plates were coated with either natural PSP94 (—●—) or GST-PSP94 (- -○- -) at 50 ng/well. For GST-PSP, amount was adjusted to equal molar 3.5:1. Standard nPSP94 protein with varying amounts as indicated on the x-axis and PSP94 polyclonal antibody (1:25,000 dilution) were preincubated together and added to the coated wells. **B:** Only GST-PSP94 was coated at 50 ng/well, and competed for PSP94 antiserum (1:25,000), preincubated with either nPSP94 (—●—) or GST–PSP94 (- -○- -) ranging as indicated on the x-axis. Secondary antibody reaction and color development were measuring the excess binding of the remaining polyclonal antibody (see Materials and Methods). Each point represents mean values of duplicate samples.

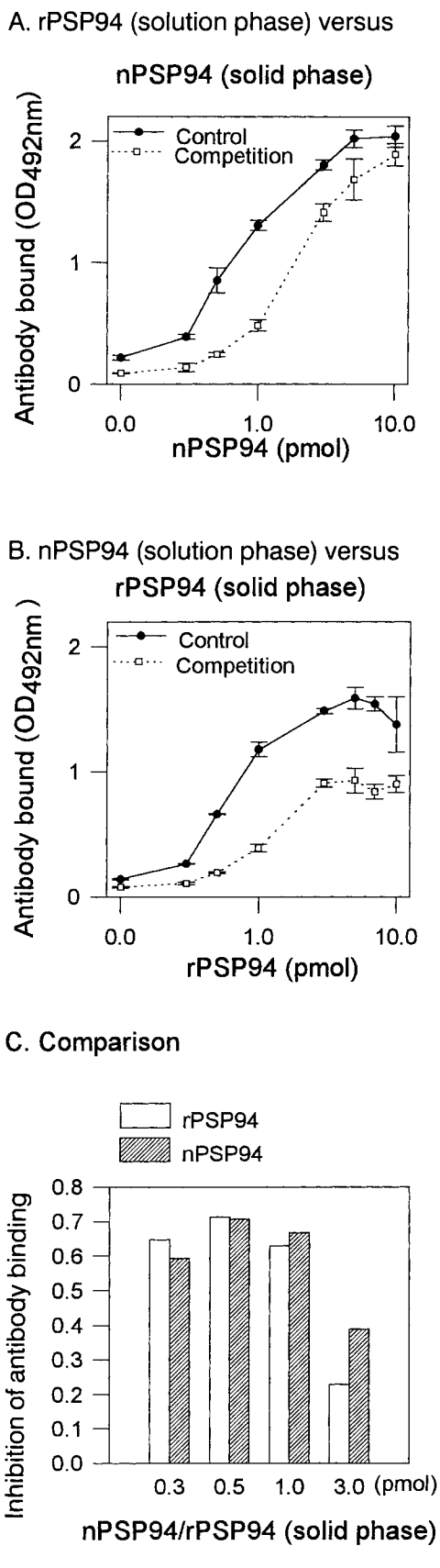


column purification. The purified PSP94 protein was used to perform the same competition as Figure 3 and was tested on an equimolar basis. The results of the reciprocal competition of the competitor against nPSP94 (as coating antigen, shown in Fig. 4A) and the competitor nPSP94 against rPSP94 (as coating antigen, shown in Fig. 4B) were similar. From low dose to nearly saturated levels (50 ng, ~5 pmol per well) of the coating proteins, both competition curves showed similar slopes. Figure 4C shows nearly equal extent of competition inhibition between the two purified PSP94 proteins from low (3–10 ng, i.e. ~0.3–1 pmol) to nearly saturated (10–30 ng, i.e. ~1–3 pmol) dosages. Comparing Fig. 3C and Fig. 4C, purified rPSP94 can inhibit 60–70% of the binding of nPSP94 with polyclonal antibody, GST–PSP94 fusion protein could only inhibit no more than 50% (Fig. 3C) in the very low dosage of the coated PSP94 protein. Crude preparation of rPSP94 showed maximum coating only at 70 ng (~7 pmol) per well. If higher than 70 ng to saturation levels, the binding was inhibited. Therefore there was no plateau of competition for nPSP94 against rPSP94 (Fig. 4B), and rPSP94 has a slightly lower affinity of binding with their antibody, if using maximum binding of control level (the plateau level of Fig. 4A,B) as standard.

Denatured, Natural, and Recombinant PSP94 Protein Retain Most of the Binding Affinity

A series of experiments were performed to test the epitope activity of both natural and GST–PSP94 proteins denatured in vitro by different reagents: 8M urea 0.1 M glycine pH 9, boiling, detergent SDS, and reducing chemical β-mercaptoethanol (2ME). Two quantities of coating protein were tested: 50 ng and 100 ng

Fig. 3. Competitive ELISA to assess the relative affinity of nPSP94 and GST–PSP94 for anti-PSP94 antibody. **A:** nPSP94 immobilized in wells (0.1–10 pmol per well) was used to capture antibody incubated for 2 h with (- -○- -) and without (—●—) competitor antigen GST–PSP94 in solution phase. **B:** GST–PSP94 immobilized in wells (0.025–25 pmol per well) was used to capture antibody incubated for 2 h with (- -○- -) or without (—●—) competitor nPSP94 in solution phase. **C:** Degree of inhibition of antibody binding was calculated on the mean values as $1-OD_{492nm}$ of competition/OD_{492nm} of control. For GST–PSP94 in solid phase and nPSP94 in solution (□) and nPSP94 in solid phase and GST–PSP94 in solution (▨), were calculated for 4 different amounts of coating antigen (0.1, 0.2, 0.3, 1 pmol), using data presented in A and B. Error bars of the points are mean values from triple samples ± SD.



per well, representing the mid-log and saturation point of the dose response to PSP94 polyclonal antibody shown in Figure 3. Both natural and GST–PSP94 proteins, denatured by high concentrations of urea and alkali solution (8 M urea, 0.1 M glycine, pH 9.0), retained the same binding ability (in solid phase) with polyclonal antibody (Fig. 5). After treatment of low concentration of detergent and reducing agent (1% SDS/0.01% β-mercaptoethanol), the binding activity of nPSP94 also remained unchanged. GST–PSP94 was sensitive to the reducing agent treatment, as it lost most the binding ability after the treatment. Comparing Figure 5A and B, GST–PSP94 showed resistance to boiling denaturation, in contrast to nPSP94.

Location of the Linear Epitope

In order to confirm and locate the linear epitopes of PSP94 antibody, two peptides (N30 and C28) representing two ends of the PSP94 protein sequence were used to test binding with PSP94 antiserum. Figure 6A shows that C-terminus of PSP94 protein (C28 peptide) has very low binding activity with PSP94 antiserum, while the N-terminal peptide (N30) shows significantly higher binding affinity with the PSP94 antibody. Two quantities of 50 ng and 100 ng, i.e., 10 and 20 pmol of the coating peptide, were tested and the results were the same. Competitive ELISA (Fig. 6B) experiments were performed using natural, GST–PSP94, and rPSP94 protein (in the solution phase), to compete with the N30 peptide coated in the plastic well (solid phase). The results show that all sources of PSP94 protein, natural and rPSP94 proteins in the solution phase can compete and inhibit N-terminal peptide immobilized in solid

Fig. 4. Competitive ELISA to assess the relative affinity of nPSP94 and rPSP94 crude preparation for anti-PSP94 antibody. **A:** nPSP94 immobilized in wells (0.1–10 pmol per well) was used to capture antibody incubated for 2 h with (- -○- -) and without (—●—) competitor antigen rPSP94 in solution phase. **B:** Crude preparation of rPSP94 immobilized in wells (0.025–25 pmol per well) was used to capture antibody incubated for 2 h with (- -○- -) or without (—●—) competitor nPSP94 in solution phase. **C:** The degree of inhibition of antibody binding was calculated on the mean values as $1-OD_{492nm}$ of competition/OD_{492nm} of control. For rPSP94 in solid phase and nPSP94 in solution (□), and nPSP94 in solid phase and rPSP94 in solution (▨), were calculated for four different amounts of coating antigen (0.3, 0.5, 1, 3 pmol), using data presented in A and B. Error bars of the points are mean values from triple samples ±SD.

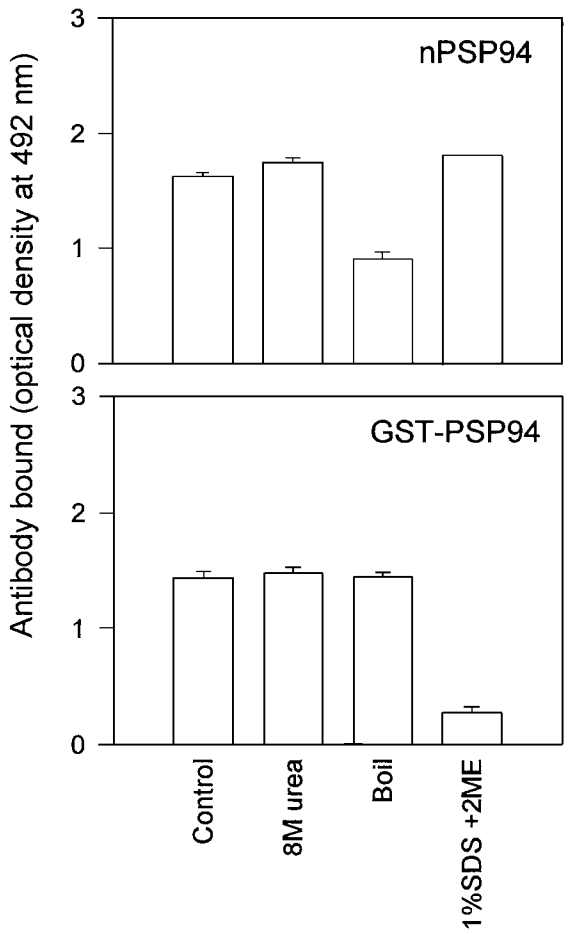


Fig. 5. Direct ELISA to assess the ability of nPSP94 (A) and GST–PSP94 protein (B) denatured in vitro by various conditions, to bind with anti-PSP94 antibody. Denaturing procedure and direct ELISA were conducted as described in Materials and Methods. Values are mean of triplicate measurements ±SD.

phase binding with anti-PSP94 antibody. The maximum inhibition using 5 pmol (50 ng) PSP94 protein or the equivalent of GST–PSP94 (~200 ng) to compete with 50 ng (~15 pmol) of N30 peptide, i.e., at three times less in mole mass, was about 30%.

DISCUSSION

PSP94 has shown promise as a potential prostate cancer marker to monitor the course of prostate cancer therapy, especially after anti-androgen treatment [Vaze et al., 1979; Doctor et al., 1986; Dube et al., 1987; Tremblay et al., 1987; Abrahamsson et al., 1988; Teni et al., 1989; Huang et al., 1992, 1993]. Two hypotheses have been proposed to explain the presence of PSP94 in patients with prostate cancer. PSP94 may act as a tumor suppressor [Sheth et al., 1988; Lokeshwar et al., 1993; Mundel et al., 1993; Garde et al., 1993a] or as an IgBF [Liang

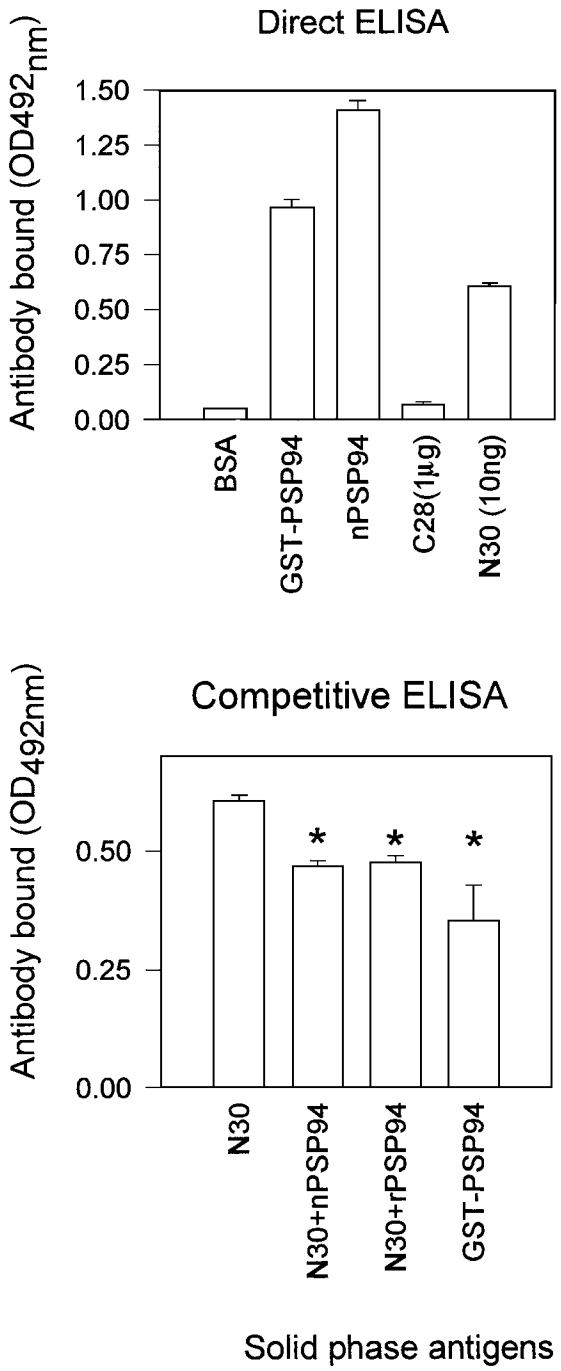


Fig. 6. Assessment of the binding activity with anti-PSP94 antibody using chemically synthesized peptides representing N- and C-termini of PSP94 protein sequence. Direct ELISA (*top*) was conducted to test immobilized N30 and C28 peptides (either 50 or 100 ng, i.e., ~15 pmol or 30 pmol per well) the ability to be recognized by polyclonal antibody. Competitive ELISA (*bottom*) was performed with the immobilized N30 peptide (50 ng, i.e., ~15 pmol) against competitor proteins: 5 pmol (~50 ng) of natural protein, 5 pmol (~175 ng) of GST–PSP94 fusion protein and 5 pmol (~50 ng) of the crude preparation of rPSP94. Error bars are mean values from triple samples ±SD. Inhibition of three sources of PSP94 proteins to N-terminal peptide (N30) binding with the polyclonal antibody was tested statistically significant ($P < 0.05$), as indicated by asterisk (*).

et al., 1991, 1992; Maeda et al., 1993]. PSP94 has been measured in blood samples from prostate cancer patients and has been shown as a tumor marker by many researchers, although with different explanation [Sheth et al., 1987; Tremblay et al., 1987; Teni et al., 1988, 1989; Hara et al., 1989; Huang et al., 1992, 1993; Garde et al., 1993b; Maeda et al., 1994]. On the other hand, as PSP94 is high in concentration in seminal plasma (1,320 ± 183 μg/ml) [Dube et al., 1987], a function with respect to reproductive biology has been proposed: either as a sperm coating antigen [Johansson et al., 1984; Akiyama et al., 1985; Hara et al., 1989] or as an immunosuppressive factor (IgBF) [Liang et al., 1991, 1992; Maeda et al., 1993]. These two proposed functions raised the question of the antigenic structure of PSP94. The expression of sperm coating antigens is central to the consideration of their immunogenicity in the reproductive tract. As an immunosuppressive factor (IgBF), PSP94 would protect the spermatozoa from attack by the immunosurveillance system of the female reproductive tract. We propose that the epitope structure should be identical, since PSP94 acts as an antigen to two kinds of antibodies—polyclonal or monoclonal—against nPSP94 protein purified from seminal plasma, and CD16 series monoclonal antibodies against type III IgG receptor.

This article describes the use of a GST–PSP94 protein to study the epitope structure of PSP94 protein. Results of both Western blotting and competitive ELISA indicate that GST–PSP94 can be used to assess PSP94 levels and function in clinical material. Recombinant PSP94 has only one extra amino acid (Gly) located before the mature protein excision site in the N-terminus. The entire GST–PSP94 protein showed the same, or only slightly lower, ability binding with a polyclonal antibody against natural PSP94 as compared with nPSP94. In determining the standard curves of immunoassay by competitive ELISA of natural and GST–PSP94 using GST–PSP94 as coating antigen (Fig. 2A), similar slopes were obtained, indicating that GST–PSP94 and nPSP94 have epitopes recognized with similar affinity and binding kinetics by the polyclonal antibody. While using GST–PSP94 as standard antigen in both the solid and solution phases, a steeper slope than the control (nPSP94 as competitor antigen in the solution phase) was obtained (Fig. 2B). In most of the dosages tested for solution phase antigen GST–PSP94 (competitor standard), there were always more free antibodies (epitopes) left after competition, giving higher B/B0 values than those of the nPSP94. Therefore the slope difference of the two curves indicates the differences of the affinity and the epitopes recognized. Experiments of reciprocal, competitive ELISA between natural and GST–PSP94 fusion protein confirmed this analysis (Fig. 3). As summarized in Figure 3C, when the competitor antigen in solution phase at lower than 1 pmol, both proteins showed comparable competition inhibition, however, when at higher than 1 pmol, nPSP94 showed stronger affinity with PSP94 antibody. We propose that this difference is due to the steric hindrance of the carrier protein GST, 2.5 times larger in size and has another four cysteine residues, and part of the epitopes of rPSP94 has been wrapped inside. This explanation was supported by reciprocal competition binding test between natural and crude preparation of the purified rPSP94 protein. The results (Fig. 4) shows comparable inhibition on a molar basis against the solid phase antigen at a lower (0.1–0.5 pmol), till nearly saturated range (~ 3 pmol). Our results of competition ELISA, either self or reciprocal competition, demonstrate the linear epitope structure of PSP94 protein, since all the recombinant PSP94 proteins tested were denatured.

PSP94 is a small molecule (10,770 Da), with 10 cysteine residues (more than 10%) in the secreted mature protein. Our experimental data have shown the difficulties in correctly folding in vitro, even in vivo inside an *E. coli* cell, into the same conformation as the natural protein. Recombinant GST-PSP94 has very poor solubility and most of the rPSP94 protein is in the inclusion body fraction inside the *E. coli* bacteria cell. This is typical of the difficulties in correct folding. When we purified GST–PSP94 fusion protein from the inclusion body, which was dissolved in a strong denaturing solution (8 M urea, 0.1 M glycine pH 9.0), quickly desalted and renatured in nondenaturing condition, we were actually screening the soluble form for both carrier GST and rPSP94 protein resulted from a self-renaturing process. The relative yield was low, with only less than 5% of the whole GST–PSP94 protein trapped in the inclusion body fraction recovered. Purified recombinant PSP94 protein showed inability to properly bind onto the plastic well (Fig. 4B), since it showed decreased binding activity after saturated coating (7 pmol), indicating that it was possibly in a different conformation from that of the natural form of PSP94. It is also possibly due to this

reason that rPSP94 showed lower inhibition binding with antibody in competitive ELISA with nPSP94 when tested at a dosage of higher range of the coating antigen (> 3 pmol, Fig. 4C).

Our results also demonstrate that nPSP94 protein is resistant to denaturing agents (urea, base), detergent and reducing chemical (1% SDS 0.01% β-mercaptoethanol) without changing any detectable affinity to PSP94 polyclonal antibody as shown in quantitative ELISA (Fig. 5). The chemicals used in this test dissociated either the hydrogen bonds or disulfur bonds, which were the major organic bonds maintaining the secondary and super structure of the whole PSP94. Since nPSP94 as an antigen is a single-chain protein without glycosylation [for review, see Hara et al., 1989], our in vitro denaturing tests suggest that the correct folding of 10 cysteine residues in nPSP94 protein contributes little to the epitope activity of PSP94. The epitope of PSP94 appears to reside mostly in the primary, rather than secondary or even super structure of PSP94. The epitope activity in recombinant GST–PSP94 protein also showed resistance to urea and base treatment, indicating that PSP94 protein can sustain repeated urea/base treatment, as GST–PSP94 protein had already been denatured by urea/base treatment during the process of purification. In contrast to nPSP94, the function of the epitope of GST–PSP94 was sensitive to reducing reagent treatment, probably due to the overcross-linking of cysteine residues with those in carrier protein GST. Consistent with this explanation, crude preparation of rPSP94 also showed resistant to β-mercaptoethanol treatment (data not shown).

Computer analysis (PC-GENE program, IntelliGenetic, Inc., CA) of the predicted secondary structure of PSP94 showed three high points of hydrophilicity, two of which are located at the C-terminus (79–85, 69–74) and one at the N-terminal end (12–17). In an attempt to locate a linear epitope, two peptides representing N- and C-termini were synthesized and tested. The results of immunoassay indicate that C-terminus (C28 peptide) is not an immunoactive area (Fig. 6). It is unlikely that this result is due to the host factors in the specific epitope selection, since this result has been repeatedly obtained from different immunized rabbits (data not shown) and was also reported previously by other researcher [Mbikay et al., 1988]. ELISA shows that the N-terminal (N30) peptide has antigenic activity. The polar distribution of the epitopes may result from the fact that the highly charged and basic C-terminus has been buried in the cross-linked, correctly folded protein. The binding affinity of the PSP94 antiserum to N30 peptide is competitively inhibited by rGST-PSP, indicating that GST–PSP94 is immunoactive in the N-terminus. This result supports the hypothesis of a linear epitope structure of PSP94 antigen. Competitive ELISA between N-terminal peptide and PSP94 proteins also indicates that the epitope may reside in the N-terminal region. We are currently performing systematically the peptide mapping, using synthetic peptides and GST–PSP94 fragments with the objective of proving that the N-terminus is the immunodominant area of PSP94 protein.

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