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Purification of prostate-specific membrane antigen using conformational epitope-specific antibody-affinity chromatography

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Abstract

Prostate-specific membrane antigen (PSMA) is a type II membrane protein that has attracted significant attention as a target for immunioscintigraphic and radioimmunotherapeutic applications for prostate cancer. However, definitive studies on its substrate and inhibitor specificity as well as protein–protein interactions have been somewhat limited by difficulties in the purification of native PSMA. In this study, we optimized the purification of native PSMA from LNCaP cells using conformational epitope-specific antibody-affinity chromatography. Western blot analysis and an HPLC-based enzymatic activity assay were used to compare the yield and activity of PSMA purified by different methods. The ratio of purified PSMA in a native and active conformation was determined by quantifying the amount of non-native PSMA not retained in a second antibody-affinity isolation. The addition of both a neutralization step and the inclusion of Zn^{2+} to the equilibration buffer in desalting step provides considerable enhancement in the yield of active PSMA from LNCaP cells.

Keywords: Prostate-specific membrane antigen, PSMA; Conformational epitope; Antibody-affinity chromatography

Prostate-specific membrane antigen (PSMA)[1] is a 750 amino acid, type II membrane glycoprotein with a molecular weight of approximately 100–120 kDa dependent upon the glycosylation pattern. Its structure includes a cytoplasmic domain (amino acids 1–19), a transmembrane domain (amino acids 20–43), and an extracellular domain (amino acids 44–750) responsible for its enzymatic activity. Belonging to the M28B peptidase family, PSMA possesses two known enzymatic activities: *N*-acetylated-alpha-linked-acidic dipeptidase (NAALADase) and folate hydrolase [1–4]. Although the exact role of PSMA in prostate carcinogenesis and progression is still unknown, its high expression in prostatic cancer and cellular surface presentation mark PSMA not only as a prognostic indicator, but also as an excellent target for prostate cancer imaging [5,6]. For example, radiolabeled anti-PSMA monoclonal antibodies and small molecules have been tested for the imaging of prostate cancer with encouraging results [7–9].

Aggregation or denaturing problems commonly encountered during the purification and storage of membrane proteins remains a challenge in obtaining PSMA in a native and active conformation. Other researchers have avoided such challenges by using recombinant DNA techniques to express only the extracellular domain of PSMA with an "affinity tag" to purify recombinant PSMA in a subsequent step [10,11]. Although recombinant PSMA studies have provided insights into its structural and enzymatic characterization, the absence of the cytoplasmic and transmembrane domains [12] along with altered glycosylation patterns are expected to affect its physical or enzymatic properties [10,13]. Furthermore, the lack of distinction between properly folded and denatured or misfolded protein as a consequence of affinity tag-based chromatography imposes a limit upon definitive substrate and inhibition studies of PSMA.

Herein, we describe an optimized purification of PSMA from LNCaP cells using conformational epitope-specific

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[1] *Abbreviations used:* PSMA, prostate-specific membrane antigen; NAALADase, *N*-acetylated-alpha-linked-acidic dipeptidase; TFA, trifluoroacetic acid.

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antibody-affinity chromatography. The LNCaP cell line was established from a biopsy of a supraclavicular lymph node in a 50-year-old Caucasian male with metastatic prostate carcinoma [14]. Using both Western blot analysis and enzymatic activity assays, we demonstrate the effectiveness of this method on both the yield and enzymatic activity of native PSMA.

Materials and methods

Cell culture

LNCaP cells (ATCC-CRL-1740) were obtained from the American Type Culture Collection (Manassas, VA), and grown in a complete growth medium (RPMI 1640 containing 10% heat-inactivated fetal calf serum (FBS), 100 U of penicillin and 100 μg/mL streptomycin). The cells were maintained in a 95% air, 5% CO_2 humidified incubator at 37 °C. Cells were prepared in T-75 flasks and after four days of growth, the media was replaced and the cells were grown for another three days prior to harvesting.

Purification of PSMA

Based upon our preliminary procedure, three methods were designed to optimize PSMA purification procedure from LNCaP cells [15]. The following buffers were used in the experiments described herein: buffer A (phosphate-buffered saline containing 0.1 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol), buffer B (50 mM pH 7.5 Tris containing 150 mM NaCl), buffer C (buffer B containing 0.1 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol), buffer D (100 mM glycine, 150 mM NaCl, pH 2.5), buffer E (1 M KCl, 150 mM NaCl), buffer F (50 mM pH 9.0 Tris containing 150 mM NaCl), buffer G (100 mM pH 11.5 glycine containing 150 mM NaCl), buffer H (1 M pH 7.0 Tris), and buffer I (buffer B containing 1% Triton X-100 and 5 mM $ZnCl_2$).

Crude membrane protein preparation

Twelve flasks of cells were grown to confluence, the media was decanted, cells were washed once in 10 mL of buffer A, and scraped in 5 mL of buffer A. The cell suspension (60 mL) was centrifuged at 1000*g* for 10 min at 4 °C to obtain a cell pellet. The pellet was homogenized (15 strokes) manually with a Dounce Homogenizer on ice in 6 mL of buffer C, centrifuged at 50,000*g* for 60 min at 4 °C, and the supernatant was discarded. The resulting membrane pellet was washed with 10 mL of buffer C, centrifuged at 50,000*g* for 20 min at 4 °C, the supernatant was discarded and this sequence was repeated a second time. The washed membrane pellet was resuspended and homogenized (30 strokes) in 3 mL of buffer B containing 2% Triton X-100 and 1X Halt™ protease inhibitor cocktail (Pierce; Rockford, IL) to solubilize membrane proteins. After a final centrifugation at 5000*g* for 10 min at 4 °C, the supernatant (membrane protein extract, 3 mL) was collected, and stored at −80 °C until use.

Conformational epitope-specific antibody-affinity chromatography

An antibody-affinity column was prepared with 1 mL of Sepharose-3C6 affinity resin (Northwest Biotherapeutics; Seattle, WA) into a Poly-Prep Chromatography Column (Bio-Rad Laboratories, Hercules, CA). Sepharose-3C6 affinity resin is a product resulting from the coupling of CNBr-activated Sepharose 4B with the anti-PSMA antibody 3C6 (Northwest Biotherapeutics; Seattle, WA) that reacts with a protein conformational epitope present in the extracellular portion of human PSMA. The column was sequentially equilibrated with 3 mL of buffer B, 3 mL of buffer D, and 3 mL of buffer B, respectively. Approximately 3 mL of crude membrane protein extract was loaded on the column and the flow through was collected and re-loaded twice. The column was sequentially washed with the following buffers: 3 mL of buffer B containing 1% Triton X-100, 3 mL of buffer E containing 1% Triton X-100, 3 mL of buffer B containing 1% Triton X-100, and 3 mL of buffer F containing 1% Triton X-100. *Method A* involved eluting PSMA from the antibody-affinity column with 1.6 mL of buffer G into a tube containing 400 μl of buffer H neutralize the eluate. The neutralized eluate was immediately loaded onto a Sephadex 10DG desalting column, which was pre-equilibrated with 10 mL of buffer I. 7 mL of buffer I was used to elute purified PSMA from the desalting column and 1 mL aliquots of eluate were collected. Typically, the fourth through seventh fractions contained purified PSMA and were pooled, mixed with glycerol (final concentration: 10%), divided into small volumes, and stored at −80 °C prior to use. *Method B* was performed in the same manner as Method A except that the eluate neutralization step was omitted. PSMA was eluted directly from the antibody-affinity column with 2 mL of buffer G into a Sephadex 10DG desalting column and the remaining steps of Method A were followed. *Method C* was similar to Method B except that the Sephadex 10DG desalting column was pre-equilibrated and PSMA was eluted from it with buffer B containing 1% Triton X-100 instead of buffer I.

Protein concentration of PSMA purified by each method was determined by the BCA method (Micro BCA™ Protein Assay Reagent Kit; Pierce; Rockford, IL) as per the manufacturer's protocol. BSA was used as the protein standard at concentrations of 5, 10, 15, 20, 25, 30, and 40 μg/mL.

Isolation of PSMA in non-native conformation

In order to determine the ratio of native to non-native conformation of PSMA after purification, PSMA purified by each of the three methods were subjected to a second antibody-affinity isolation. Affinity columns were prepared and equilibrated as described in Method A. Approximately 1 mL samples of purified PSMA were loaded on the columns. As 3C6 antibody is highly effective for binding PSMA in its native conformation, only PSMA in a native conformation was retained by the antibody-affinity column

Table 1
Comparison of methods for the purification of PSMA

Method A	Method B	Method C
For each method, PSMA was eluted from the antibody affinity resin with 100 mM pH 11.5 glycine containing 150 mM NaCl		
Neutralization of affinity resin eluate with 1 M pH 7.0 Tris.	No neutralization of affinity resin eluate.	No neutralization of affinity resin eluate.
For each method, the eluate from the antibody affinity resin was loaded onto desalting column		
Elution from desalting column with 50 mM pH 7.5 Tris containing 150 mM NaCl, 1% Triton X-100, and 5 mM $ZnCl_2$.	Elution from desalting column with 50 mM pH 7.5 Tris containing 150 mM NaCl, 1% Triton X-100, and 5 mM $ZnCl_2$.	Elution from desalting column with 50 mM pH 7.5 Tris containing 150 mM NaCl and 1% Triton X-100.

while denatured or unfolded PSMA (non-native conformations) did not bind and was collected as flowthrough.

Western blot analysis

Four microliter of each sample of purified PSMA and 10 μl of the respective flowthrough samples from the second antibody-affinity isolation were resolved on a NuPAGE™ 4–12% Bis-Tris Gel (Invitrogen, Carlsbad, CA), electrophoresed for 60 min at constant 200 Volts under reducing conditions, and then transferred to a PVDF Immobilon-P Transfer Membrane (Millipore Corporation, Bedford, MA) at 400 mA for 100 min in a transfer apparatus-Owl Bandit VEP-2 (Owl, Portsmouth, NH) according to the manufacturer's instructions. PSMA was detected by the anti-PSMA mouse antibody 4D8 (Northwest Biotherapeutics; Seattle, WA) and immunoblot analysis was performed with Protein Detector TMB Western Blot Kit (KPL, Gaithersburg, MD) following the manufacturer's instructions. These analyses were repeated thrice with similar results obtained in each case. The relative amounts of PSMA in each lane on Western blots were quantified by National Institutes of Health (NIH) Image J software (http://rsb.info.nih.gov/ij/).

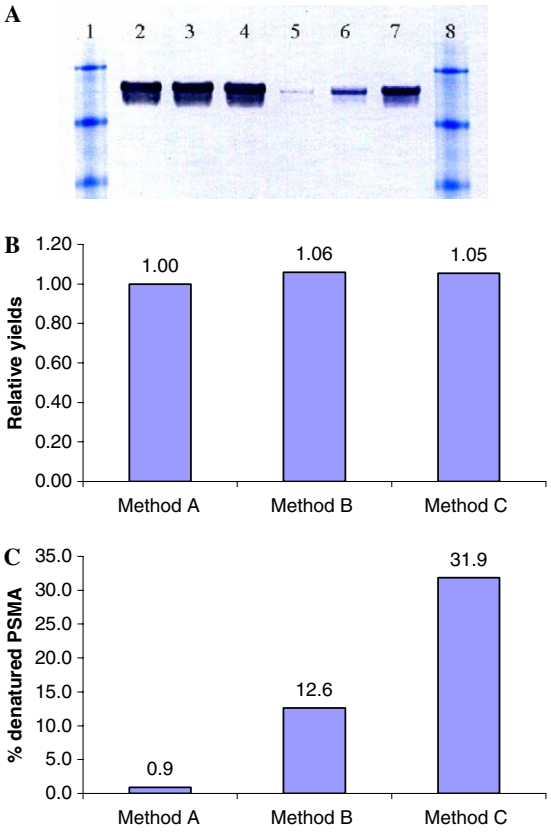

Fig. 1. Characterization of purified PSMA. (A) Western blot analysis. Lanes 1 and 8: pre-stained protein marker; lanes 2, 3, and 4: PSMA purified by Methods A, B and C, respectively; lanes 5, 6, and 7: flowthrough of second affinity chromatography for Methods A, B, and C, respectively, representing denatured or unfolded PSMA generated by each method. (B) Relative yields of PSMA for each method. (C) Percentage of denatured PSMA for each method.

Enzymatic activity assay

Enzymatic activity was determined using the HPLC-based analysis described previously [15,16] with minor modifications. The assay is based upon the separation and quantification of a gamma-diglutamate substrate and its PSMA-mediated hydrolytic product by HPLC. Briefly, purified PSMA was diluted 200-fold in 50 mM pH 7.5 Tris containing 1% Triton X-100. A substrate solution (10 μM) was prepared in 50 mM pH 7.5 Tris. To a mixture of 25 μl of diluted PSMA solution and 200 μl of 50 mM pH 7.5 Tris containing 1% Triton X-100 was added 25 μl of substrate solution to initiate the enzymatic reaction. The reaction was allowed to proceed for 15 min with constant shaking at 37 °C then terminated by the addition of 25 μl of a solution containing 2% trifluoroacetic acid (TFA) in methanol followed by vortexing. To the quenched incubation mixture

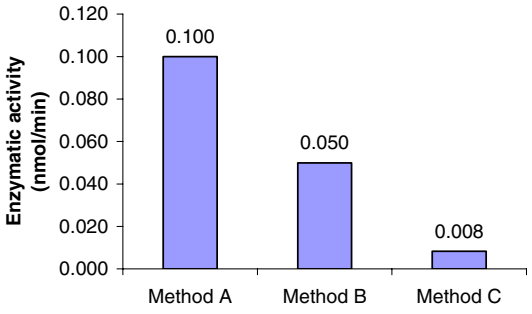

Fig. 2. Enzymatic activity of PSMA purified by Methods A, B, and C. The amount of enzyme activity for PSMA purified by each method is inversely proportional to the percentage of PSMA denatured during each method (Fig. 1C).

was added 25 μl of 0.1 M K_2HPO_4, the mixture was vortexed, then centrifuged for 10 min at 7000*g*. Aliquots (85 μl) of each sample were analyzed by HPLC as previously described [15,16].

Results and discussion

In contrast to general affinity chromatography, conformational epitope-specific antibody-affinity chromatography for the purification of native PSMA employs resin-bound anti-PSMA monoclonal antibody 3C6 that reacts with a protein conformational epitope present in the extracellular portion of human PSMA [17]. As this antibody binds PSMA when present in a native conformation, only PSMA in a native conformation is retained by the affinity resin.

Once detergent-solublized membrane proteins from LNCaP cells were obtained, the mixture was subjected to conformational epitope-specific antibody-affinity chromatography for the purification of PSMA. Our objective was to identify conditions for this purification procedure by which highly active PSMA was obtained in good yield. A comparison of the methods explored is outlined in Table 1. Western blot results demonstrated that all three methods provided similar yields of PSMA (Fig. 1A and B).

When samples from each method were subjected to a second antibody-affinity chromatography step, different ratios of natively-folded PSMA were retained (Fig. 1A and C). Method A resulted in the least amount of PSMA in a non-native conformation (0.9%) suggesting that PSMA initially purified by this method was predominately in an active conformation. These results are consistent with enzymatic activity data obtained in which PSMA purified by this method exhibited the greatest enzymatic activity (0.100 nmol/min) when compared to the other two methods (Fig. 2).

Previous studies demonstrated that the enzymatic activity of PSMA was dependent upon pH, ionic strength, and the presence of detergent [3,18,19]. Indeed, we found that 150 mM NaCl caused a considerable reduction (>90%) in enzymatic activity of purified PSMA (data not shown). The neutralization step of Method A, which distinguishes it from Method B, immediately reduced the pH from 11.5 to ~7.6 after PSMA elution from the antibody-affinity column. The low amount of denatured PSMA obtained in Method A when compared to Method B demonstrates that high pH conditions promote denaturation of PSMA. In comparison to Method B, Method C confirmed the importance of the essential Zn^{2+} cofactor during the desalting step. This result suggests that Zn^{2+} may have a stabilizing role in addition to its functional role in PSMA's enzymatic activity.

In conclusion, we have identified a protocol that is simple, rapid, and highly efficient for purifying native PSMA from LNCaP cells. As revealed by the results for Method A, both a neutralization step and the inclusion of Zn^{2+} to the equilibration buffer in a desalting step result in considerable retention of PSMA in an active conformation. Using this method, only 12 T-75 flasks of LNCaP cells were necessary to obtain 4 mL of highly purified PSMA at a concentration of approximately 60 μg/mL. By diluting this protein 200-fold for a typical incubation with substrate, we estimate that 32,000 enzymatic assays can be performed. We expect that this method is should provide sufficient amounts of PSMA for studies aimed at further characterizing this important cancer target.

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