



Optimizing the Preparation Procedure of Recombinant PSCA, as a Practical Biomarker in Prostate Cancer

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Background: The unique expression pattern of prostate stem cell antigen (PSCA) in a number of prevalent neoplasms has made the antigen a great target for cancer researches, and many clinical methods have been developed based on the application of this tumor marker. Hence, optimal PSCA laboratory production can be considered a hallmark for many researchers.

Objective: An analytical study was designed to improve the quality and quantity of PSCA production.

Materials and Methods: The effects of different compositions of lysis buffers and some ultrasound durations were assessed by calculation of the protein recovery followed by PSCA specific blotting experiments. Then, based on the results of the web-based characterization, interference removal, followed by re-solubilization of the protein in various buffers, was designed, applied, and assessed.

Results: Since the selection of an appropriate methodology depends merely on the research purposes, we tried to discuss the pros and cons of the investigated methods according to the hydrophobic nature of PSCA as well as its dramatic tendency to aggregate in the form of inclusion bodies in the expression hosts.

Conclusions: We introduced a newly designed method to fit the delicate immunological surveys and overcome some limiting factors in PSCA production.

Keywords: Extraction; Protein aggregation; Prostate stem cell antigen; Purification; Western blotting

1. Background

Specific measurable indicators of cancers, known as biomarkers, can play critical roles in identifying the nature of diseases. Hence, the scope of molecular, cellular, and imaging biomarkers is rapidly increasing through the efforts made by researchers worldwide to provide more sophisticated surrogate markers. Effective cancer biomarkers are those which can efficiently help a clinician to diagnose and monitor the disease. They also must have the ability to predict metastasis risk and suggest an appropriate therapy (1). None of the present biomarkers can ideally meet all the aforementioned characteristics, but there are a number of biomarkers used efficiently for prostate cancer (PCa) such as prostate-specific antigen (PSA), prostatic acid phosphatase (PAP), and prostate stem cell antigen (PSCA). PSA has been extensively used for the diagnosis and monitoring

of prostate cancer. Nevertheless, its low specificity may lead to a high number of false positives. PSCA is a cell surface molecular marker that has been proven to be overexpressed in several prevalent malignancies, including renal cell carcinoma, bladder, gastric and pancreatic neoplasms (2, 3).

This valuable biomarker can also be monitored in liquid biopsies via detection of circulating tumor cells (CTCs) (4). Medical application of PSCA has gained more attention in the case of prostate malignancies as not only its up-regulation has been reported in the metastatic castration-resistant prostate cancer (mCRPC) but also its over-expression has been positively correlated with Gleason score. PSCA has been proven to act as both oncogenic and tumor-suppressing agents and is also widely used for prognosis and theranostics of prostate cancer (e.g. immunotherapy) (2). Nevertheless, its

precise biochemical and physiological functions remain unclear (5, 6). Hence, PSCA has been the subject of many types of research to decipher its biological and physicochemical properties to improve the efficacy of the present clinical methods.

To investigate the dark side of biochemical, physiological, and clinical characteristics of PSCA as well as the development of medical techniques, a sufficient amount of this antigen must be provided for research purposes. Generally, recombinant-DNA technology is used in many laboratories to clone and express PSCA corresponding genes. In this way, *E. coli* is an appropriate host which has been extensively used for the production of recombinant PSCA. After expression in the host, the protein must be recovered and purified; however, this procedure does not always yield a sufficient amount of the antigen, and sometimes the quality of the product does not satisfy the researcher (7). Formation of inclusion bodies (IBs) due to overexpression of the recombinant product and its hydrophobicity, in addition to lack of post-translational modifications in the prokaryotic host, can be considered the main problem associated with the method (5, 8). Therefore, optimizing the procedure to increase the quality and quantity of the product can be of much interest, which leads to much saving in time and costs.

2. Objectives

In the present study, we investigated the effects of ten different laboratory methods to extract and purify the recombinant PSCA produced in the *E. coli* host. The investigated methods are either the commonly used ones or in some cases are newly introduced. We aimed to present a more efficient method for PSCA production at a laboratory scale and examine its physicochemical behavior accordingly.

3. Materials and Methods

3.1. Biological and Chemical Materials

The BL21 (DE3) strain of *E. coli* was purchased from Pasteur Institute of Iran and used as an expression host. The expression constructs, pET-28a(+) containing the PSCA coding region with six-histidine (6His) tag at its two termini, was provided by GenScript (USA). Miniprep kit was purchased from Vivantis Technologies Sdn Bhd (Malaysia). All the other chemicals and reagents were of analytical grade from Merck (Darmstadt, Germany), Sigma-Aldrich (Taufkirchen, Germany), Biomedicals (Meckenheim, Germany) and Invitrogen™|Thermo Fisher Scientific (Fremont, CA, USA).

3.2. Preparation of the Construct and Expression of the PSCA Recombinant Gene

The expression construct was prepared and tested according to the standard molecular biology protocols. The positive vectors were then transformed into the competent cells of *E. coli*, BL21 (DE3) strain, by thermal shocking to overexpress the recombinant PSCA protein in the Luria-Bertani (LB) medium supplemented with 50 µg.mL⁻¹ kanamycin (9, 10). The transformation was performed according to the BioLabs protocol with some modifications. The bacterial cells, carrying our recombinant vectors, were harvested by centrifugation at 1600 ×g for 20 min at 4 °C. They were then stored at -20 °C for further analysis.

3.3. Extraction and Purification of the Recombinant PSCA; Trying Different Conditions

3.3.1. Protein Extraction in Different Lysis Buffer Solutions

Five different lysis buffer solutions were prepared and applied (**Table 1**). The common RIPA buffer composed of 150 mM sodium chloride, 50 mM Tris pH 8.0, an

Table 1. The list of different lysis buffers used for recombinant PSCA extraction and their western blotting results.

| | Lysis buffers | Western blot analysis result |
|-----|---|------------------------------|
| I | RIPA (150 mM sodium chloride, 50 mM Tris pH 8.0, cocktail protease inhibitor, 0.5% sodium deoxycholate (W/V), 0.1% SDS (W/V) and 1.0% Triton X-100 (V/V)) | No signal |
| II | 0.05% 2-mercaptoethanol (V/V), 50 mM Tris.HCL pH 8.0, cocktail protease inhibitor and 2.0% Triton X-100 (V/V) | No signal |
| III | 0.05% 2-mercaptoethanol (V/V), 50 mM Tris.HCL pH 8.0, cocktail protease inhibitor and 1.0% SDS (W/V) | Faint band |
| IV | 0.05% 2-mercaptoethanol (V/V), 50 mM Tris.HCL pH 8.0, cocktail protease inhibitor and 2.0% SDS (W/V) | Sharp band |
| V | 0.05% 2-mercaptoethanol (V/V), 50 mM Tris.HCL pH 8.0, cocktail protease inhibitor and 5.0% SDS (W/V) | Sharp band |

appropriate amount of protease inhibitor cocktail, 0.5% (W/V) sodium deoxycholate, 0.1% (W/V) SDS, and 1.0% (V/V) Triton X-100, and the four others were the same in the 2-mercaptoethanol (0.05% (V/V)), Tris-HCl pH 8.0 (50 mM) and protease inhibitor cocktail concentrations but varied in detergent type and/or quantity. Accordingly, sodium deoxycholate (0.5% (W/V)), Triton X-100 (2.0% (V/V)) and SDS at 1.0%, 2.0% concentrations and 5.0% (W/V) were selected and applied. Sufficient and equal amounts of the harvested bacterial cells were then resuspended and incubated in each buffer for 20 min on ice. This extraction procedure was also assisted by an ice-cold sonication protocol (5 min with 10 s pulses during 10 s intervals) in JENCONS (130 W, UK) instrument. Finally, the extracted lysates were recovered by 15000 ×g centrifugation of the mixture at 4 °C for 15 min, and the cell debris accumulated in the pellet was discarded. The lysates were then stored at -80 °C for further analysis.

3.3.2. Protein Extraction at Different Ultrasound Exposure Durations

Ultrasound-assisted extractions were assessed in three different durations, routinely used in the laboratories (1, 5, and 10 min) (11). Hence, sufficient and equal amounts of the harvested bacterial cells were re-suspended and incubated in the most efficient lysis buffer again, from section 2.3.1, for 20 min on ice. This lysis procedure was then assisted by sonication at the mentioned durations for each sample. The ultrasound energy was applied in 10 s pulses during 10 s intervals for all the defined durations, and the JENCONS instrument used here was 130 W power, 50% amplitude, and 20 kHz frequency. The remaining steps were the same as in section 2.3.1.

3.3.3. Elimination of Non-Protein Components

After removing the cellular debris from the lysates, non-protein components of the supernatants were eliminated via an ice-cold acetone precipitation procedure. For this purpose, four volumes of pre-chilled (-20 °C) acetone was added to the samples and vortexed. They were then incubated at -20 °C for an hour. Proteins were then recovered by centrifugation at 13000 ×g for 15 min at 4 °C. Purified protein pellets were washed with a volume of pre-chilled acetone once more, air-dried, and resolubilized in an appropriate buffer (12).

3.3.4. Resolubilization of the Purified Protein Pellets in Different Buffers

The effect of re-solubilizing buffers was assessed on the most efficient procedure, from the previous sessions

(2.3.1 and 2.3.2). For this purpose, ultra-pure deionized water, phosphate-buffered saline (known as PBS), and Tris-HCl buffer containing 2% (W/V) SDS and 0.05% (V/V) 2-mercaptoethanol were used for re-solubilizing the protein pellets, recovered after the precipitation step. Complete resolubilization was checked out by centrifuging the solutions at 15000 ×g for 20 min at 4 °C, and if the resolubilization procedure was not fully obtained, the supernatant was picked up as the pure soluble protein. These purified protein solutions were stored at -80 °C for further analysis.

3.3.5. Purification of the Recombinant PSCA

Poly(His)-tagged PSCA recombinant protein was purified from the crude bacterial lysates or the total protein extracts through the His-tag affinity column (Ni-NTA agarose) according to the Invitrogen's instruction. The purified recombinant PSCA was collected and stored at -80 °C for further analysis.

3.4. Protein Assay

Protein concentrations were assessed in different steps of the procedure. Considering the diversity of the reagents and materials used and their interference with different methods of protein assay, (13) protein concentrations were measured by either UV-absorbance at 280 nm (in a NanoDrop 2000c spectrophotometer, Thermo Scientific) or BCA assay (using a Pierce BCA Protein Assay Kit, Thermo Scientific).

3.5. Examination of the Purified Recombinant PSCA: Assessment of Different Procedures

The quality and quantity of poly(His)-tagged recombinant PSCA was assessed by a wet western blot system. For this purpose, equal amounts of protein samples (obtained from each procedure) were separated on 12% SDS-PAGE mini-gels (Bio-Rad), according to Laemmli's protocol (5, 8). Finally the poly(His)-tagged recombinant PSCA visualized via a luminescent reaction which was recorded on a Kodak radiology film.

3.6. Computer-Assisted Sequence Analysis

The biophysicochemical properties of recombinant PSCA sequence and its native form as well as some other well-known proteins were calculated with the aid of ExPASy online tools. Hence, the protein sequences were used to generate the GRAVY (grand average of hydropathy) score values via ExPASy ProtParam server (<http://web.expasy.org/cgi-bin/protparam/protparam>). The hydrophobicity plots of the poly(His)-tagged recombinant PSCA and its native sequence were also traced by Kyte and Doolittle algorithm using ExPASy

ProtScale server (<http://web.expasy.org/cgi-bin/protscale/protscale/>) to broaden the information. Finally, all the obtained data were recorded to further discuss the results of the experimental sections.

4. Results

In an analytical *in vitro* study, to improve the quality and quantity of the recombinant PSCA protein production, ten different methods were applied and evaluated. These methods summarized in **Figure 1** were either the well-established, commonly used methods or the newly

introduced ones for PSCA production, designed based on the behavior of biophysiochemical protein in the experiments.

4.1. Confirmation of Successful Expression of PSCA Fusion Protein

Before starting the optimization procedure, the successful expression of PSCA fusion protein after IPTG induction was confirmed by simple SDS-PAGE analysis. The results of this confirmation test (**Fig. 2A**) showed overexpression of a protein band near

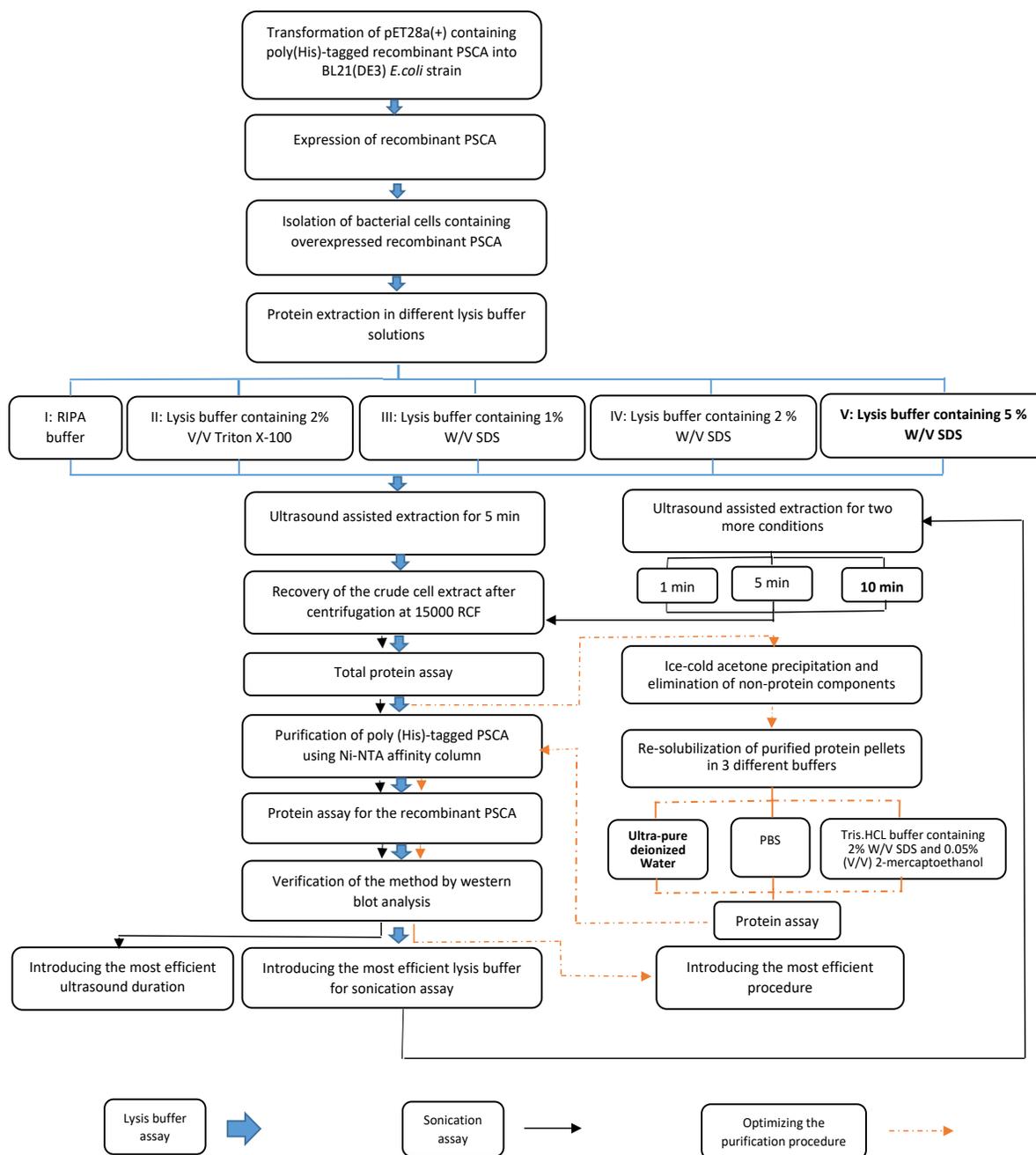


Figure 1. Summarized ten different methods used for the PSCA production and purification.

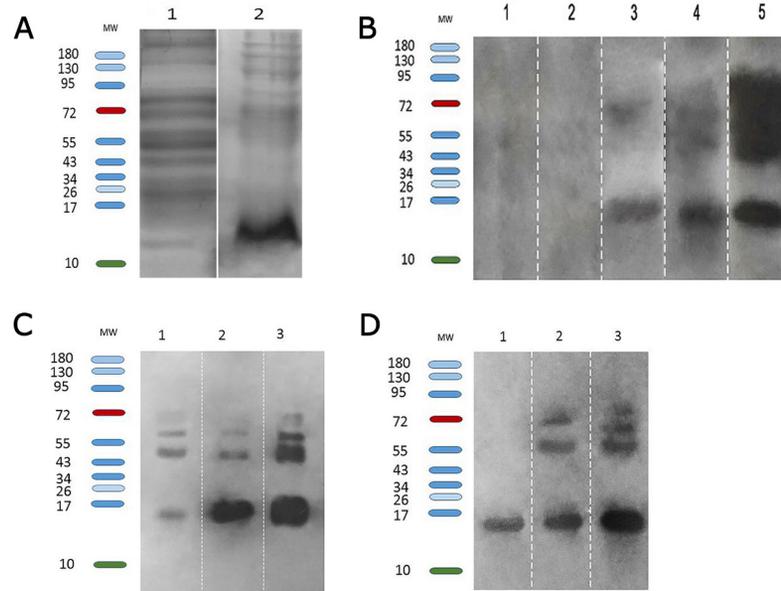


Figure 2. A: Results of SDS-PAGE analysis for recombinant PSCA expression before (lane 1) and after (lane 2) IPTG induction. B: Results of the western blot analysis, indicating the effect of the application of different lysis buffer solutions to obtain the crude cell extract from the E. coli cells. Lanes 1, 2, 3, 4, and 5 correspond to the I, II, III, IV, and V lysis buffer solutions (Table 1), respectively. C: Results of the western blot analysis showing the efficacy of the three different sonication durations. Lanes 1, 2, and 3 correspond to 1, 5, and 10 minutes sonication time, respectively. D: Results of the western blot analysis indicated the efficiency of the protein pellet resolubilization in three different solubilization buffers. Lanes 1, 2, and 3 are protein pellets resolubilized in distilled water, PBS, and Tris-HCL buffer containing 2% SDS (W/V) and 0.05% 2-ME (V/V), respectively.

the molecular weight of about 15.0 kDa, which corresponded to the predicted size of the poly(His)-tagged recombinant PSCA.

4.2. Analysis of Five Different Lysis Buffer Solutions Effect on the Yield of the Recombinant PSCA

We started to optimize the yield of the recombinant PSCA extraction via a survey on the effect of five different lysis buffer solutions. As mentioned in section 2.3.1, the buffers were varied mainly in the detergent type and/or quantity. The results of these assays are depicted in Table 1, and Figure 2B shows that in the case of I and II buffers, no detectable PSCA bands could be observed; however, in the three remaining buffer solutions, PSCA specific bands appeared with different intensities. It can be deduced that in contrast to high concentrations of SDS, sodium deoxycholate, Triton X-100 and low concentration of SDS (0.1% (W/V)) failed to extract PSCA from bacterial lysates. Thus, strong detergent treatments are needed to extract PSCA from bacterial lysates. A positive correlation of the intensity of the PSCA specific band with the increased concentration of SDS, as a powerful ionic detergent, in the lysis buffer solutions confirms our idea. Moreover, the Western blot analysis, total protein yields as well

as purified PSCA protein yields were also assessed for each of these five experiments (Table 2, part A). The data for these assessments indicated various results for the blotting analysis. Hence, strong detergent treatment not only increases the total protein yields (from 5.16 0.82 $\mu\text{g}\cdot\text{mL}^{-1}$ to 9.13 0.36 $\mu\text{g}\cdot\text{mL}^{-1}$) but also affects the yields of purified PSCA extraction (from 0.650.13 $\mu\text{g}\cdot\text{mL}^{-1}$ to 1.100.12 $\mu\text{g}\cdot\text{mL}^{-1}$).

4.3 Analysis of Different Ultrasound Durations Effect on the Yield of the Recombinant PSCA

The most efficient lysis buffer solution was then applied to investigate the effect of different ultrasound durations on the yield of the recombinant PSCA extraction. The results of this assessment (Fig. 2C) indicated that increasing the duration of the ultrasound-assisted extractions from 1 to 10 min not only increased the intensity of PSCA specific bands in the blotting profiles but also significantly increased the total (from 5.1 0.12 $\mu\text{g}\cdot\text{mL}^{-1}$ to 10.5 0.22 $\mu\text{g}\cdot\text{mL}^{-1}$) and PSCA protein (0.57 0.21 $\mu\text{g}\cdot\text{mL}^{-1}$ to 1.25 0.31 $\mu\text{g}\cdot\text{mL}^{-1}$) concentrations (Table 2, part B).

4.4. Enhancing the Purity of the Recombinant PSCA

A noteworthy point in all of these seven experiments

Table 2. Recovery of recombinant prostate stem cell antigen from *E.coli* after each assay, A; lysis buffer assay, B; sonication assay, C; solubilization buffer assay.

| | Lysis buffer | RIPA buffer | Lysis buffer containing 2% V/V Triton X-100 | Lysis buffer containing 1% W/V SDS | Lysis buffer containing 2% W/V SDS | Lysis buffer containing 5% W/V SDS |
|---|---|-----------------|---|------------------------------------|------------------------------------|------------------------------------|
| A | Protein concentration before affinity purification (mg.mL ⁻¹) | 5.16 ± 0.82 | 3.75 ± 1.00 | 6.24 ± 0.52 | 7.66 ± 0.73 | 9.13 ± 0.36 |
| | Protein concentration after affinity purification (mg.mL ⁻¹) | 0.65 ± 0.13 | 0.71 ± 0.22 | 0.89 ± 0.09 | 0.96 ± 0.20 | 1.10 ± 0.12 |
| B | Ultrasound exposure time | 1 min | 5 min | 10 min | --- | --- |
| | Protein concentration before affinity purification (mg/ml) | 5.1 ± 0.12 | 8.3 ± 0.09 | 10.5 ± 0.22 | --- | --- |
| | Protein concentration after affinity purification (mg.mL ⁻¹) | 0.57 ± 0.21 | 0.98 ± 0.25 | 1.25 ± 0.31 | --- | --- |
| C | Medium | Distilled water | PBS | SDS buffer | | |
| | Protein concentration after re-solubilization (mg.mL ⁻¹) | 2.18 ± 0.03 | 3.36 ± 0.06 | 5.49 ± 0.04 | --- | --- |
| | Protein concentration after affinity purification (mg.mL ⁻¹) | 0.35 ± 0.06 | 0.53 ± 0.03 | 0.86 ± 0.05 | --- | --- |

(five experiments for the assessment of lysis buffer solutions and two more for the ultrasound durations) is the presence of nonspecific bands (**Fig. 2B and Fig. 2C**), far from the expected PSCA molecular weight (about 15.0 kDa). To get rid of these nonspecific unexpected bands and enhance the purity of the product, three more experiments were designed and performed. The newly introduced methods were based on the elimination of non-protein interfering components via an ice-cold acetone precipitation procedure followed by re-solubilizing the protein pellets in water as well as the two other appropriate buffers (PBS and Tris-HCl containing 2% (W/V) SDS, as mentioned in section 2.3.4). As it is depicted in **Figure 1**, these alterations were applied on the most efficient procedure from the previous sessions (lysis buffer and ultrasound assays) just before purification of poly(His)-tagged PSCA via Ni-NTA affinity column. The results of checking the complete resolubilization of protein pellets confirmed the successful resolubilization of proteins in the Tris-HCl buffer, which was supplemented with a high concentration of SDS. In contrast, water and PBS buffer failed to completely resolubilize the pellets, and as expected, this failure was more obvious in the performance of pure water. These effects were also reflected in the results of the protein assay investigations (**Table 2, part C**). Therefore, as the total

protein and PSCA concentrations (2.18 0.03, 0.35 0.06, 3.36 0.06 (mg.mg⁻¹) and 0.53 0.03, 5.49 0.04, 0.86 0.05 (mg/ml) for water, PBS and Tris-HCl containing 2% (W/V) SDS respectively) increased, the polarity of the resolubilization buffer decreased. The most interesting feature of these three investigations was observed in the results of their western blot analysis (**Fig. 2D**), which occurred by complete elimination of the unexpected protein bands in water. These results were also accompanied by increased intensity of PSCA predicted and/or unexpected bands by decreasing the polarity of the resolubilization buffer.

5. Discussion

PSCA is a glycosylphosphatidylinositol (GPI)-anchored cell membrane glycoprotein with 123-amino acid and is located on the outer surface of the cell membrane lipid rafts; special structures which are enriched in cholesterol, glycosphingolipids, and other lipidated proteins (**Fig. 3**). To discuss and interpret the results obtained here, we decided to assess the biophysicochemical properties of this Jekyll and Hyde molecule. Hence, computer-assisted sequence analysis was performed via ExPASy online tools according to section 2.6. The results of this analysis indicated that although the comparison of the GRAVY index for native PSCA protein (0.48) and the recombinant poly(His)-tagged PSCA (0.12)

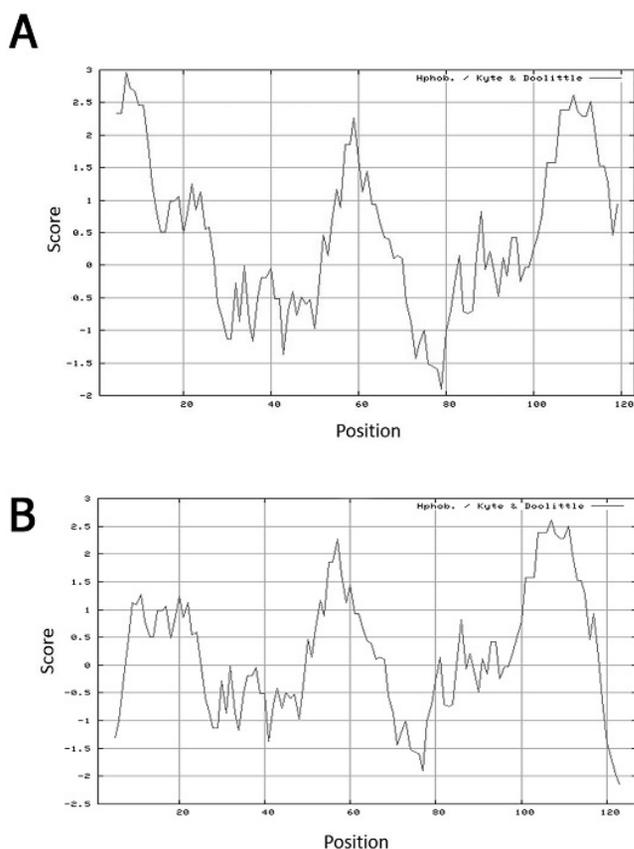


Figure 4. A; The hydrophobicity plot of PSCA in native form, B; The hydrophobicity plot of recombinant poly(His) tagged PSCA.

of the protein) (17). Further, overexpression of the recombinant protein will also result in improper protein folding, and a lack of post-translational modifications will increase the instability and hydrophobicity of the protein (18). Thus, there is a lot of evidence that suggests that the expression of recombinant poly(His)-tagged PSCA will be accompanied by the formation of inclusion bodies (IBs) in bacterial cells (5, 19, 20).

In the assessment of the effect of five different lysis buffer solutions, we showed that strong detergent treatments were needed to extract the recombinant PSCA from the expression host and solve the problem of negative results. This can be attributed to the tendency that originated from the hydrophobic nature of the fusion protein to precipitate with the hydrophobic cell debris as well as the membrane fragments (5, 21, 22). Hence, replacement of the mild detergent conditions by high concentrations of SDS, as a powerful ionic detergent, would result in the protein extraction from the precipitated cellular debris into a soluble fraction (23). Results of the protein assays would also confirm the idea. Similarly, increased power of the ultrasound-assisted extraction via a prolonged application time

can lead to an increased PSCA predicted band and concentration. This can be attributed to the hydrophobic nature of the recombinant protein, as well. In a polar ionic environment, ultrasound-assisted extraction facilitates momentary detachment of the hydrophobic protein compartment from the cellular debris, allowing the formation of the solvation layer and increasing the protein yield in the aqueous sections (24, 25).

Another noteworthy point in the experiments associated with the lysis buffer and ultrasound assays was the presence of unpredicted bands. The blotting analysis indicated that the intensities of these unpredicted bands would also increase by increasing ultrasound power and the detergent (**Fig. 2B** and **Fig. 2C**). Since these nonspecific bands vanished when the pure protein contents were resolubilized in water in the last three experiments, we can deduce that the bands are strongly hydrophobic IBs, formed during the recombinant PSCA expression and extraction. It should be remembered that in the aforementioned experiment, complete resolubilization in water was negative and, the supernatant was picked up as the pure soluble protein in the blotting assay. Therefore, increasing the detergent and ultrasound power not only increased the PSCA specific and predicted band but also could help in more efficient isolation of IBs. Many other reports introduce sonication as a fast, simple, and inexpensive mechanical cell rupture method, which can also be used for isolation of IBs from bacterial cells, and confirms the results presented here (5, 26). Nonetheless, results obtained here suggest that strong detergent power and prolonged ultrasound durations can help to increase the recombinant PSCA yields and unwanted IBs aggregates. Here, we designed some additional experiments to overcome the problem of IBs nonspecific bands. We specifically precipitated the total protein content obtained from the extraction procedure not only to reach the optimal purity of the product but also to replace the lysis buffer with other media which conversely push the more hydrophobic aggregates out of the solution and yield pure recombinant PSCA compartments. The present study introduced a more efficient procedure for the PSCA protein recovery and enhancing its purity based on the interpretation of the biophysicochemical behavior of the protein. The results obtained here showed that the hydrophobicity of the protein, in addition to its great tendency to accumulate in the form of insoluble IBs, strongly affected the outcomes of the experiments. We also introduced a method to eliminate the nonspecific aggregated protein bands by complete elimination of the unwanted

aggregates in the highly polar and hydrophilic medium of water. Providing a sufficient amount of purified cancer biomarkers is very important for clinicians and researchers; hence, it facilitates the experimental and industrial process for the development of diagnostics tools (e.g. molecular imaging techniques) as well as biomarker-based drug development approaches (e.g. production of recombinant protein-based vaccines or other kinds of immunotherapy). Finally, it was shown that the newly introduced approach yielded a suitable product for the researchers working on the delicate investigations, including those with immunological or pharmaceutical nature.

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