

# Per Aqueous Liquid Chromatography (PALC) as a Simple Method for Native Separation of Protein A

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**Abstract** Staphylococcal protein A (protein A) is an important protein frequently used in research studies within the fields of biomedicine and biotechnology. Due to some limitations in available protein purification methods which can hold the native structure of the protein A without changing the folding or adding histidine to structure of this protein, its separation in the native form is difficult. In this study, a new cost-effective and powerful technique was introduced for separation of the full-length and truncated forms of recombinant protein A, without any alteration in their 3D

structures. Per aqueous liquid chromatography with bare silica gel stationary phase and water:acetonitrile as the mobile phase was proved to be an attractive choice among the range of separation methods. Similar to hydrophilic liquid chromatography, this method employs high percentage of water in mobile phase. The effects of mobile phase composition, pH, and salt concentration on the retention behavior of protein A on bare silica gel stationary phases were investigated. In this method, applying high amounts of aqueous solvent accompanied by a minimum percentage of organic solvent could successfully separate protein A with preservation of folding, and any affinity-tagged group such as histidine has not occurred on its structure. Purity of the fractions obtained by the proposed method was confirmed using SDS-PAGE, western blotting, and matrix-assisted laser desorption ionization time-of-flight mass spectrometry. According to the results of ELISA, separated proteins retained their ability of binding to antibody.

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**Keywords** Protein A · Staphylococcal protein A · Per aqueous liquid chromatography (PALC) · Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF)

## Abbreviations

PALC	Per aqueous liquid chromatography
Protein A	Staphylococcal protein A
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
MALDI-TOF MS	Matrix-assisted laser desorption ionization time-of-flight mass spectrometry
Fc	Fragment crystallizable
IgG	Immunoglobulin G

IMAC	Immobilized metal ion affinity chromatography
HILIC	Hydrophilic interaction liquid chromatography
ACN	Acetonitrile
TFA	Trifluoroacetic acid
BSA	Bovine serum albumin
PBS	Phosphate buffer saline

## Introduction

Staphylococcal protein A (protein A) is prevalently used in a wide range of biotechnological and biomedical applications. Protein A binds to the fragment crystallizable (Fc) portion of all human antibody subtypes (except IgG3) and certain types of antibodies from other species, such as pig, dog, rabbit, goat, and mouse [1]. It may be coupled to a solid support (e.g., silica gel, agarose, cellulose or cross-linked dextran) in an affinity chromatography resin to purify antibodies, proteins, and peptides [2, 3]. The protein A is frequently used in routine laboratory methods including agglutination, ELISA, western blotting, and many other well-known applications. It is also employed in a growing number of therapeutic applications. Accordingly, protein A has been found to bind to immunocomplexes and immunoglobulins from blood and serum, and inhibit certain autoimmune diseases, such as rheumatoid arthritis and thrombocytopenic purpura [4]. Therefore, purified protein A is very important in many different applications.

Several methods have been used to purify the protein A from high-density fermentation samples. These generally include ion-exchange chromatography [5, 6], affinity chromatography [7, 8], and gel filtration [7]. Due to low resolution of ion-exchange and gel-filtration chromatographic techniques in purification procedures, these methods may not be suitable for therapeutic applications of protein A. On the other hand, reagents used in the affinity chromatography technique are not economical, due to high cost and low binding capacity. Moreover, disadvantages of HIS-tag system in affinity chromatography are its impact on expression levels of the protein [8]. In principle, despite the relatively small size and charge of the poly-histidine affinity tags, the interference between affinity tag and the protein function is likely [9]. In addition, a common problem when using poly-histidine affinity tags for protein purification is nonspecific binding of untagged proteins on ionic immobilized affinity columns (i.e., Ni<sup>2+</sup> immobilized beds), because elimination of poly-histidine-tagged proteins would be a time-consuming process. Although histidine occurs relatively infrequently (only 2% of all protein residues are histidine), some cellular proteins contain two or more adjacent histidine residues [10]. These proteins

have an affinity for the immobilized metal ion affinity chromatography (IMAC) matrix and may be co-eluted with the protein of interest, resulting in significant contamination of the final product, and hence making it useless for therapeutic applications. Disulfide bond formation between the protein of interest and other proteins can also lead to contamination. However, application of 2-mercaptoethanol buffers generally eliminates this potential problem. Nonspecific hydrophobic interactions can also cause copurification with the desired protein [11]. Despite the wide utility of hydrophilic liquid chromatography in analysis of amino acids and peptides, it has rarely been employed for the separation of intact proteins. Only a few number of biomolecules including lysozyme, BSA, and chymotrypsinogen have been separated using HILIC [12]; nevertheless, their biological functions and 3D structures might be effected under high percentages of organic solvents, such as alcohols (e.g., isopropanol or methanol) and acetonitrile as the mobile phase [13–15]. The same problem is also encountered in reversed-phase (RP) chromatography [16, 17]. It seems that limitations of HILIC for separation of intact proteins are related to ionic and H-bond interactions between the protein A and surface of the stationary phase; in a way that it might be difficult to elute them using low percentages of water under HILIC conditions. With regard to this fact that electrostatic interactions as well as large number of H-bond donor and acceptor moieties play a key role in retention of proteins on polar stationary phases [18], applying a suitable amount of water in HILIC mode (aqueous-rich condition), for elution of them with reasonable resolution would be considered as an appropriate method for separation of intact proteins.

Per aqueous liquid chromatography (PALC) in framework of green analytical chemistry has attracted much attention in analysis and separation of polar compounds [18, 19]. This mode of chromatography is also known as reverse hydrophilic interaction liquid chromatography, because of replacing the hazardous organic solvents and reagents with safe ones [20]. Features of PALC have been investigated in recent years and it is consequently proposed as an alternative to HILIC method for separation of some analytes.

During previous studies, the full-length and truncated forms of protein A were cloned, expressed, and secreted into the culture medium. Expression was optimized via changing various parameters [21–24]. To the best of our knowledge, there is not any report on the application of PALC for protein purification procedures. In this study, attempts were made to purify the commercially important protein A using this cost-effective and powerful separation technique. This chromatographic approach offers several advantages including simple operation conditions, high purity, shorter purification times, and maintaining correct folding and function of the protein, compared to the previously described methods.

## Materials and Methods

### Chemicals and Reagents

HPLC-grade acetonitrile (ACN) was obtained from Chemlab NV (Belgium). Trifluoroacetic acid (TFA), ammonium acetate, and all other chemicals were purchased from Merck (Darmstadt, Germany). All chemicals were of analytical-grade purity. Water was obtained via double distillation and further purified using Milli-Q system.

### Biological Samples

Two extracellular proteins secreted by *Escherichia coli* were included in this study: One of the samples contained the full-length form and the other encompassed the truncated form of recombinant protein A. Both recombinant samples were cloned and expressed in *E. coli*, as reported previously [24].

### High-Performance Liquid Chromatography and MALDI-TOF Mass Spectrometry

HPLC system (Knauer, Germany) used in this study was equipped with a K-1001 pump (Knauer, Germany), K-2008 PDA detector (Knauer, Germany), and a 20- $\mu$ L injection valve. The columns used for the purpose of separation included a bare silica gel column (250  $\times$  4.6 mm, YMC, 10  $\mu$ m, 300 Å); elution was performed on the column and kept at 25 °C with the following mobile phases: A, H<sub>2</sub>O containing 0.5 mM ammonium acetate with pH value of 5.5 and B, water/acetonitrile (10/90 v/v) containing 0.5 mM ammonium acetate with pH value of 5.5. A flow rate of 1 mL min<sup>-1</sup> was applied for the analyses. The employed elution program started at 95% A and remained at this point for 5 min before changing to 85% A over 15 min. UV detection for these samples was performed at 280 nm and Analytical RP-HPLC was carried out using a Knauer C18 column (150  $\times$  4.6 mm, 5  $\mu$ m, 300 Å; Berlin, Germany) at a flow rate of 1 mL min<sup>-1</sup> with solvent A (100% ddH<sub>2</sub>O/0.1% TFA, v/v) and solvent B (90% acetonitrile/10% ddH<sub>2</sub>O/0.08% TFA, v/v/v). Both forms of protein A were separated with linear gradients of solvent B between 5 and 100% at 2% min<sup>-1</sup>, and remaining at 100% B for a further 5 min, which eventually returned to 95% A in 10 min for re-equilibration. Similarly, the UV detection for these samples was performed at 280 nm.

In confirmatory method, MALDI-TOF mass spectrometry was applied. For this purpose, equimolar samples of full-length and truncated protein A were mixed 1:1 (v/v) with saturated sinapic acid solution (20 g L<sup>-1</sup> matrix in 100% acetone/0.1% trifluoroacetic acid, Agilent technologies, Palo Alto, USA) and applied directly onto a polished steel target plate (Scout 384, Bruker Daltonics GmbH,

Bremen, Germany), and left to air-dry. Samples of protein A were analyzed and recorded on a Bruker ultraflex MALDI-TOF reflector mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany), equipped with a nitrogen laser ( $\lambda = 337$  nm). Mass spectra were recorded in positive mode using the linear mass analyzer. Calibration was initially performed via external calibration using a low MW protein standard (Bruker Daltonics GmbH, Bremen, Germany). A mass spectrum of the enzyme *N*-glycosidase F (34.5 kDa, Roche, USA) was recorded and served as a reference. Data analysis was carried out by *m/z* (Knexus Ed. vers. 2002.10.01, Genomic Solutions, Ann Arbor, MI, USA).

### SDS-PAGE Analysis and Western Blotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed, according to the Laemmli procedure [25]. Concentrations of the used resolving and stacking gels were 12 and 5% (w/v), respectively. Polyacrylamide gel was first fixed for 120 min in a solution containing 50% (v/v) methanol, 12% (v/v) acetic acid, and 5.05% (v/v) formalin. It was then rinsed for 20 min with a fixer containing 10% (v/v) methanol and 5% (v/v) acetic acid. Sensitization was carried out by treatment with 20% (w/v) sodium thiosulfate (Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>) for 2 min. The gel was then stained by immersing in 0.2% (w/v) silver nitrate (AgNO<sub>3</sub>) and 0.076% (v/v) formalin, which was then shaken for 20 min. The image was developed in a 6% (w/v) sodium carbonate (Na<sub>2</sub>CO<sub>3</sub>) solution containing 0.0004% (w/v) sodium thiosulfate (Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>) and 0.05% (v/v) formalin. Image development was terminated by repeated washes in deionized water [26].

Low molecular weight markers obtained from the Sino-American Biotechnology Company were used and western blots were developed using the IgG anti-goat HRP-conjugated antibody (Sigma, USA).

For polyclonal antibody production, purified proteins were injected into rabbits four times at 10-day intervals. At first injection, protein concentration was 300–500  $\mu$ g mL<sup>-1</sup> with the same volume of complete adjuvant. Subsequent injections contained protein concentrations of 200–300  $\mu$ g mL<sup>-1</sup> with the same volume of incomplete adjuvant, which were subsequently followed by the third and fourth subcutaneous injections. On 44th day, total blood was collected and sera were tested against purified protein and optimal dilution of anti-amylase. Sera were then used for western blot analysis at established dilution of 1:4000.

For western blot analysis, protein samples were subjected to SDS-PAGE, and then transferred to a nitrocellulose membrane (Roche, Germany). Next, the membrane was incubated with primary rabbit polyclonal antibody. As the secondary antibody, IgG anti-goat HRP-conjugated antibody (Sigma, USA) was used and the blot was developed with 4-chloro-1-naphthol substrate (Sigma, USA). It should be noted that

throughout all experiments regarding expressed proteins, equal amounts of protein were used.

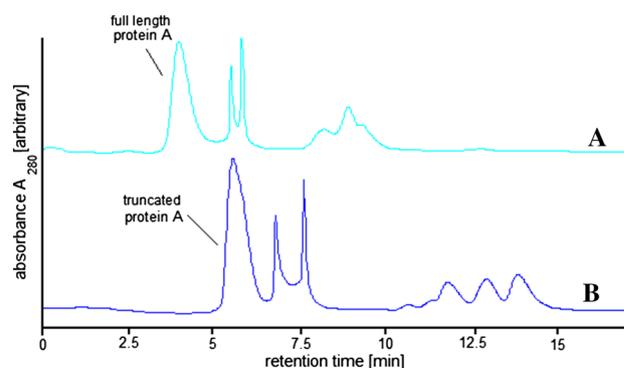
### Indirect ELISA for Detection of Protein A Forms and Functional Confirmation

Two different forms of protein A were determined using indirect ELISA [27] and their biological functions were confirmed after purification. 96-well plates (Costar, USA) were coated with protein A. For this purpose, the protein A coating-ELISA (PAC-ELISA) method was used, but with minor modifications [28]. Briefly, protein A was diluted in 100 mM carbonate buffer [3.03 g  $\text{Na}_2\text{CO}_3$  and 6.0 g  $\text{NaHCO}_3$  in 1 L of distilled water (pH 9.6)] and added to plates at a final concentration of 10  $\mu\text{g}/\text{mL}$ . These were then incubated overnight at 4 °C. After two washes with PBS, plates were incubated with 100  $\mu\text{L}$  of rabbit IgG (10  $\text{mg mL}^{-1}$ ) for 2 h at 37 °C in 50 mM  $\text{NaHCO}_3$ - $\text{Na}_2\text{CO}_3$  (pH 9.6). Subsequently, plates were washed twice with 0.1% Tween-20 in PBS and the wells were blocked overnight at 4 °C with 200  $\mu\text{L}$  of 1% BSA in PBS. After six washes with PBS, plates were finally incubated with goat IgG-conjugated horseradish peroxidase (HRP) at a 1:2000 dilution in PBS at room temperature for 30 min. Plates were eventually washed again and 100  $\mu\text{L}$  of OPD substrate solution was added to each well. After stopping the reaction with 50  $\mu\text{L}$  of 1 M  $\text{H}_2\text{SO}_4$ , the absorbance at 490 nm was determined using a microplate reader (ELX800, Biotech, USA). Functions of the two forms were then compared using independent *t* test. This experiment was repeated three times.

### Results and Discussion

A typical baseline separation of both truncated and full-length forms of protein A was achieved on silica gel column using the PALC method (Fig. 1).

According to the principle theory of separation in HILIC describing the partitioning of analyte between the mobile phase and water-rich layer partially immobilized on the stationary phase, hydrophobic analytes would be less solubilized in water-rich layer and hence show lower retention times on silica gel column. On the other hand, with regard to the key role of electrostatic and H-bonding interactions in the retention of proteins on polar stationary phases [18], highly organic conditions of HILIC buffers limit the application of this chromatographic approach for protein separation. Therefore, in the case of truncated and full-length forms of protein A, hydrophobic properties calculated by Prot-Scale software and proposed method of Parker et al. [29] (less solubilized on water-rich layer and less interaction with silica gel column), and hydrophilic residues (hardly eluted from the polar surface when using low percentages of water under

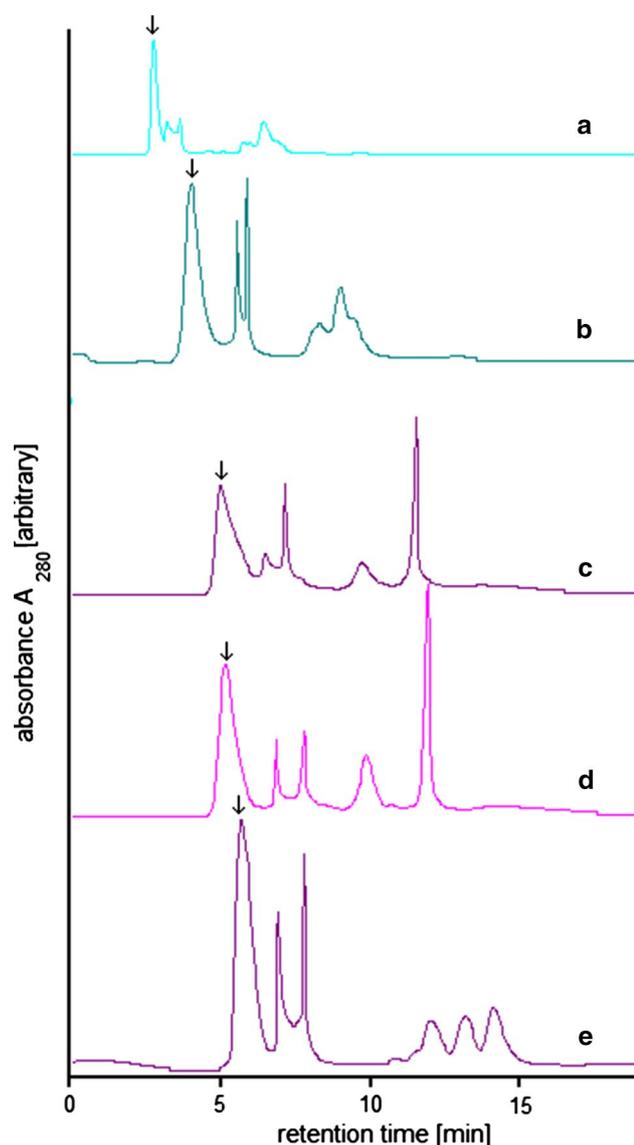


**Fig. 1** Chromatograms of full-length (A) and truncated (B) of protein A on bare silica gel (250 × 4.6 mm, 5  $\mu\text{m}$ , 100 °A). Conditions: solvent A;  $\text{H}_2\text{O}$  0.5 mM ammonium acetate with pH value of 5.5 and solvent B; 90% ACN + 10%  $\text{H}_2\text{O}$  containing 0.5 mM ammonium acetate with pH value of 5.5, gradient eluent: 0–5 min, 95% A, 5–15 min, 85% A. UV, 280 nm; The column temperature: 25 °C. Flow rate: 1  $\text{mL min}^{-1}$

HILIC conditions) led us to optimize the HILIC condition with an appropriate ratio of water to organic solvent. Hence, the PALC method employing high percentages of water was proposed as an alternative to HILIC mode to overcome the mentioned problems [12, 13] for the retention of protein A under HILIC condition as well as other proteins. Figure 1 illustrates different retention times for each form of protein A on silica gel column using an aqueous-rich mobile phase. The full-length form of protein A with more hydrophobic property was more retained on silica gel surface than the other form.

### Effect of Water Content on Retention

In the following, the truncated form of protein A was selected to investigate the PALC properties of the silica stationary phase and clarify the mechanism of separation in this method. For that, the effect of volume fractions of water in mobile phases (85, 87.5, 90, 92.5, and 95%) on protein A retention were studied. According to the results of HPLC (Fig. 2), the truncated protein A exhibited typical PALC behaviors of increasing retention with increasing water content in the mobile phase on the silica gel. It showed that the retention of the protein A was extremely sensitive to any small variation of ACN content in water-rich eluents, which could lead to the drastic composition change of the adsorbed eluent multilayer onto stationary phase. Moreover, partitioning equilibrium among 2 phases shifted toward hydrophobic layer on silica gel surface (Fig. 2). Therefore, it could be concluded that the significant retention was because of the formation of hydrophobic interactions between the siloxane groups on the surface of silica gel particles and targets with lower



**Fig. 2** Comparison of Protein A (truncated form) separation on bare silica gel ( $250 \times 4.6$  mm,  $5 \mu\text{m}$ ,  $100^\circ\text{\AA}$ ) using different ratios of water in the beginning of elution: **a** 0–15 min 85% A, **b** 0–5 min, 87.5% A, changes to 85% A over 10 min, **c** 0–5 min, 90% A, changes to 85% A over 10 min, **d** 0–5 min, 92.5% A, changes to 85% A over 10 min, **e** 0–5 min, 95% A, changes to 85% A over 10 min. Conditions: solvent A;  $\text{H}_2\text{O}$  containing 0.5 mM ammonium acetate with pH value of 5.5 and solvent B; 90% ACN + 10%  $\text{H}_2\text{O}$  containing 0.5 mM ammonium acetate with pH value of 5.5. The truncated protein A peak is indicated by arrow in chromatograms

polarities (Fig. 2). As well as previous study [20]; where Pereira et al. confirmed that amino acids with lower polarities, such as leucine, isoleucine, and proline, were more retained in PALC than polar amino acids, such as glutamic acid (possessing two carboxyl side chains) and lysine (containing two amine side chains). This result is in accordance with retention mechanism of caffeine in PALC mode [20].

**Table 1** Retention time (min) of protein A at different buffer pH values

Compound	Retention time (min) at different buffer pH values				
	3.0	3.5	4.5	5.5	6.5
Protein A	3.9	4.0	4.7	5.1	6.6

Mobile phase: ACN/0.5 mM ammonium acetate(95/5, v/v). Column temperature:  $25^\circ\text{C}$ . Flow rate:  $1.0 \text{ mL min}^{-1}$ . UV detection: 280 nm,  $t_0 = 2.1$  min

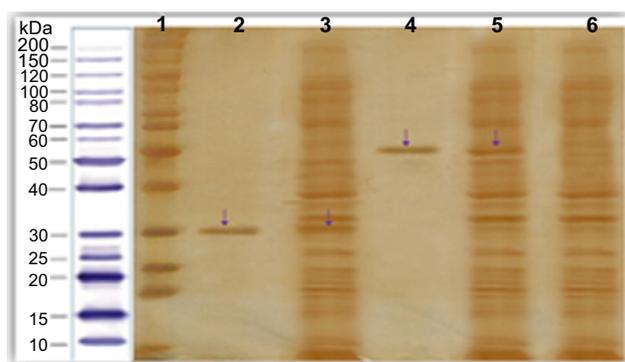
### Effect of Mobile Phase pH on Retention

Mobile phase pH plays an important role in retention and selectivity in LC by influencing solute ionization in the eluents. In current study, the effect of pH on separation was also evaluated for further investigation of the mechanism of PALC mode. Table 1 shows that the effect of the mobile phase pH on the PALC retention by changing the pH of ammonium acetate aqueous solutions at 6.5, 5.5, 4.5, and 3.5, while keeping the concentration of ammonium acetate constant at 0.5 mM. The retention time of protein A with  $\text{pI} = 5.5$  increased in the pH range from 3.5 to 6.5, the reason might contribute to be more protonation of protein A in low pH and less hydrophobicity. However, increasing the pH value from 5.5 to 6.5 leads to increase in the retention time, which could be in result of increasing the hydrophobicity of salute due to increasing the electrostatic repulsion between unprotonated salute and stationary phase (see electronic supplementary material Fig. S1).

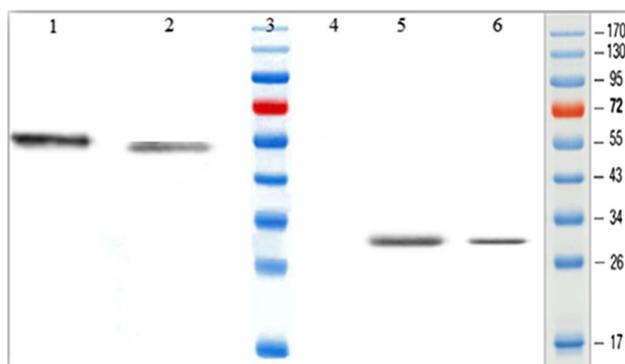
Chromatogram of truncated form was completely reproducible as well as full-length form (data are not shown). After collection of each fraction, they were separately analyzed using MALDI-TOF mass spectrometry, which proved that the purified sample was protein A. MALDI-TOF displayed masses of 32.219 kDa and 52.312 kDa for truncated and full-length protein A, respectively; a little lower than the predicted theoretical masses from DNA sequence of truncated (32.817 kDa) and full-length (52.812) forms of protein A, but within the error limit of the technique.

Collected samples were subsequently visualized on a silver-stained polyacrylamide gel (Fig. 3), which showed that both forms of the protein were separated from regions in the vicinity of peak 1. It should be noted that silver-staining procedure was applied in this study because of its high sensitivity.

Also, western blot assay for both forms of protein A confirmed that collected products from peak 1 were protein A (Fig. 4). For a more precise result, the protein obtained from peak 1 was dissolved in acetonitrile/water (5/95 v/v) and was subsequently injected onto a monolithic C18 column, followed by elution using the gradient program, as mentioned in experimental section above. It was noticed that regardless



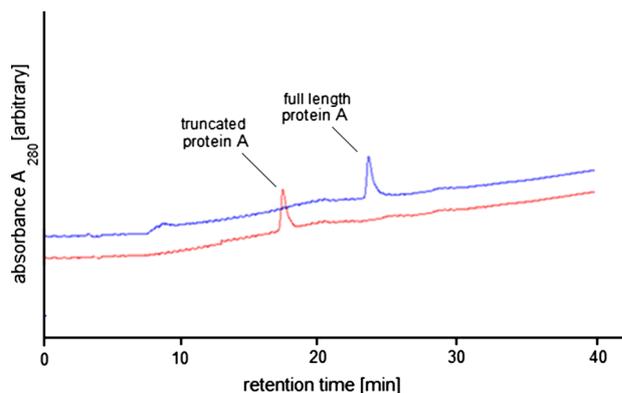
**Fig. 3** Polyacrylamide gel electrophoresis stained with silver nitrate showing the prepared protein A (full-length and truncated forms). Lane 1: protein molecular weight marker; lane 2: purified truncated protein A; lane 3: non-purified truncated protein A; lane 4: purified full-length protein A; lane 5: non-purified full-length protein A; lane 6: total protein at  $T_0$  (before induction)



**Fig. 4** Confirmation of purified protein A by western blotting technique. Rabbit serum and goat anti-rabbit IgG conjugated with HRP were used as the primary and secondary antibodies. Lane 1: the full-length form of purified protein A; lane 2: full-length form of non-purified protein A in culture supernatant (as positive control); lane 3: protein molecular weight marker; lane 4: sample before induction, at  $T_0$  (as negative control); lane 5: full-length form of purified protein A; lane 6: truncated form of non-purified protein A secreted into the medium (as positive control)

of the used solvent and/or sample concentrations only one peak appeared on the chromatogram (Fig. 5), representing a purified sample. This single peak for the full-length and truncated forms appeared at 25 and 29 min, respectively; showing an appropriate interaction between the protein and the column. Since the full-length protein has an extra hydrophobic region compared to the truncated form, it takes longer for it to come out of the hydrophobic column.

After confirming the purity of the protein (which was the protein of interest), ELISA technique was carried out as previously mentioned and protein A (each form of the protein) was attached to the bottom of the well. His-tagged protein A bought from Sigma-Aldrich Company (Sigma,



**Fig. 5** Chromatograms of obtained from peak 1; full-length (blue) and truncated (red) of protein A on a C18 column (150 × 4.6 mm, 5 μm, 300 Å; Knauer, Germany) at a flow rate of 1 mL min<sup>-1</sup> with solvent A (100% H<sub>2</sub>O/0.1% TFA, v/v) and solvent B (90% ACN/10% H<sub>2</sub>O/0.1% TFA, v/v/v). Conditions: 0–5 min, 95% A, in the following a linear gradients of solvent B between 15 and 65% at 0.66% min<sup>-1</sup>, including post-gradient equilibration steps. Similarly the UV detection for these samples was performed at 280 nm

USA) was used as positive control. This procedure consequently revealed that separation steps did not affect protein IgG-binding ability.

Statistical analysis was carried out using *t* test for mean values obtained from ELISA reaction related to the purified full-length and truncated forms, positive and negative controls. A significant difference was observed between these groups, indicating that ability of the prepared proteins (both forms) to bind to the antibody was increased, compared to the positive control protein. Each experiment was repeated three times.

## Conclusion

In the present study, both forms of protein A were separated in PALC (reverse HILIC) chromatographic mode, using high percentages of aqueous solvent which was critical for maintenance of the correct folding and function of the protein. Also, this method could eliminate the formation of disulfide bonds of interested protein with other proteins, and overcome nonspecific binding of untagged proteins to IMAC stationary phases due to their histidine residues in poly-histidine-tagged protein purification method. In addition, simplicity, speed, and cost-efficiency of separation and its potential for industrialization make it a suitable method for purification of protein A.

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### Compliance with Ethical Standards

**Conflict of interest** The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript, apart from those disclosed. No writing assistance was utilized in the production of this manuscript. The authors declare no conflicts of interest.

**Ethical approval** All applicable international, national, and institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants or animals performed by any of the authors.

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