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Immunoaffinity chromatographic isolation of prostate-specific antigen from seminal plasma for capillary electrophoresis analysis of its isoforms

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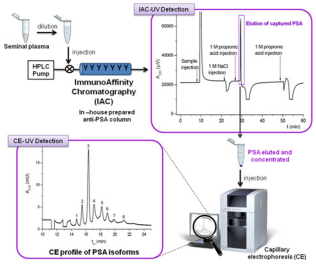
HIGHLIGHTS

- Purification of prostate-specific antigen (PSA) is performed from seminal plasma.
- Only sample dilution is required prior to immunoaffinity chromatography (IAC).
- IAC on an in-house fabricated HPLC anti-PSA column takes about 20 min.
- Elution with propionic acid does not alter the CE profile of PSA isoforms.
- IAC recovery and purity allows CE analysis of PSA isoforms from 5 mL seminal plasma.

GRAPHICAL ABSTRACT



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ABSTRACT

Prostate-specific antigen (PSA) concentration in serum has been the biomarker employed for prostate cancer diagnosis in the last two decades. However, new more specific biomarkers allowing a better differentiation of cancer from non-malignant prostate diseases are necessary. Glycosylation of PSA gives rise to different forms of the protein which can be separated into several isoforms by analytical techniques, such as CE. Because PSA glycosylation is influenced by pathological conditions, the CE pattern of PSA isoforms could be different in prostate cancer than in non-malignant prostate diseases. To study this CE pattern of PSA, prior purification of the protein from the biological fluid is mandatory. In this study an immunoaffinity chromatography method which allows PSA purification without altering the CE pattern is developed. An in-house prepared column produced with commercial anti-PSA antibodies is employed. The use of 1 M propionic acid as elution agent provides higher than 40% recovery of high purity PSA. CE analysis of PSA immunopurified from seminal plasma of a healthy individual shows the same 8 peaks as the commercially available PSA standard. Sample preparation only requires dilution with phosphate buffered saline prior to immunoaffinity purification. High repeatability for the sample preparation step was achieved (RSD% for percentage of corrected peak area in the range 0.6–5.3 for CE analysis of three independently purified seminal plasma aliquots compared to range 0.8–4.9 for a given aliquot analyzed three times by CE). IAC of five microliters seminal plasma provided enough PSA to achieve signal/noise ratio larger than 5 for the smallest CE isoforms.

Abbreviations: Ab, antibody; BPH, benign prostatic hyperplasia; fPSA, free PSA; IAC, immunoaffinity chromatography; ID, internal diameter; PBS, phosphate buffered saline; PEEK, polyetheretherketone; PSA, prostate-specific antigen; tPSA, total PSA.

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1. Introduction

Prostate-specific antigen (PSA) is a glycoprotein of about 28 kDa molecular weight, with 8% carbohydrate content corresponding to a single glycan chain attached at Asn-45, and with isoelectric point in the range 6.2–7.5 [1]. PSA is mainly produced in the prostate gland and secreted to the seminal plasma, where it is found at a concentration ranging from 0.2 to 5.5 mg mL^{-1} [2]. It can reach the blood circulation where around 70–90% of the glycoprotein is found as PSA complexed with protease inhibitors, mainly with alpha 1-antichymotrypsin (ACT) forming a complex of around 80–90 kDa molecular weight [3]. The rest of PSA in blood can be found as free PSA (fPSA), which corresponds to several molecular forms such as fragments from active PSA or proPSA and its truncated species [4]. Total PSA (tPSA) includes complexed PSA and fPSA.

The level of tPSA in serum has been approved by the US Food and Drug Administration (FDA) as biomarker for screening and monitoring of prostate cancer (PCa) [5], due to the increased concentration of the glycoprotein in blood under this condition. However, the established threshold of 4 ng mL^{-1} to discriminate between cancerous and healthy states [6] lacks specificity since other pathologies of the gland, such as benign prostatic hyperplasia (BPH), also produce a rise of PSA in serum [7,8]. This lack of specificity leads to an elevated number of false positives and finally to an excessive number of unnecessary biopsies [9]. Actually, the influence of PSA screening on prostate cancer mortality is clearly controversial [10,11]. Whereas recent studies in the European Union conclude that the PSA screening significantly reduces mortality from prostate cancer [12–14], the studies performed in North America indicate no improvement in survival for prostate cancer patients [15,16]. In order to improve the specificity of PSA as biomarker, different molecular forms of the glycoprotein, such as complexed PSA [17], fPSA [18,19], or proPSA [20,21] have been studied.

Different molecules of PSA exist due to either changes in the polypeptidic chain (proPSA, mature PSA, nicked PSA, *etc.*) [3,4,22] or variations in the glycosidic moiety. The different molecules of the glycoprotein are called forms, while each peak, band or spot separated by an analytical technique is termed isoform. Each isoform can include more than one form.

Glycosylation of proteins is known to be dependent on the physiopathological conditions, and namely to be related to prostate cancer [1,23]. Studies to correlate changes in PSA glycosylation with prostate diseases have been carried out by different techniques such as mass spectrometry, HPLC, lectin affinity, or gel electrophoresis [1,24–29]

Using two-dimensional electrophoresis in gel (2-DE), several isoforms of PSA, differing in isoelectric point and/or molecular weight, have been separated [30]. Recently, some of these isoforms have shown to be in different proportions in sera from BPH patients than in sera from prostate cancer patients and they have also shown to correlate with the prostate cancer stage [31].

CZE is an adequate technique for the separation of isoforms of intact glycoproteins of clinical interest [32]. In the case of PSA, this technique is able to separate several isoforms of the standard glycoprotein [33]. In a work recently developed in our laboratory, a total of 8 or 9 isoforms of PSA were resolved by different CZE methods [34]. These methods could be used to compare CE profiles of PSA from patients with PCa versus PSA from patients with non-malignant prostatic diseases, similarly to the above mentioned studies carried out by conventional gel electrophoresis [31]. Comparison of CE profiles has shown the potential role of the CE profiles of alfa 1-acid glycoprotein as biomarker of vascular diseases [35].

To obtain the CE profile of PSA, prior isolation of the protein from the biological sample is mandatory. The presence of interferents, such as other proteins that have tendency to adsorb on the capillary wall [36], could distort or even prevent the separation of these isoforms. In most of the cases, the clinical sample of interest is blood, because of its extraction is simple and semi-invasive, but PSA concentration in this fluid is very low (in the subnanomolar range), preventing its analysis using UV detection. Studies of PSA forms and glycosylation in PCa and benign diseases have been also performed in other biological fluids, such as urine [25], seminal plasma [27], or expressed-prostatic secretion (EPS) [37]; the last two fluids are of special interest due to the high concentration of PSA present in them and because these fluids are thought to be more reflective of the physiological state of the nearby prostate. Most of the biological fluids present a high content and diversity of proteins; in the case of seminal plasma, more than 900 different proteins account for 35–55 g L^{-1} of average protein concentration [38]. For this reason, high selective isolation techniques are needed prior to the CE analysis of intact PSA isoforms. Several affinity methods for the isolation of PSA from different matrices have been developed. Some of these methods combine several purification steps based on chromatography, including cation- exchange, size-exclusion, and thiophilic-interaction [39–41]; these methods are useful for handling large volume samples to prepare large quantity of PSA but are not adequate to our aim as they are labor intensive and require more than one day to be performed. Looking for highly selective purification methods for PSA, immunoaffinity has been carried out using magnetic beads [42] or low pressure chromatography [43–47]; these methods are slow with elution times of up to 15 h. In some instances, immunoaffinity purification has been combined with up to five chromatographic steps, increasing the length of the purification process [48]. Immunoaffinity chromatography (IAC) performed in HPLC format is an adequate tool to purify proteins because it adds the advantages of speed, controlled flow rate, and easy detection to the very high specificity of the interaction between antibody (Ab) and antigen [49–53]. To study the CE profiles of isoforms of PSA purified from biological samples, the purification method should not alter PSA in such a way that its pattern would be modified. None of the above-mentioned methods studies the effect of purification on the PSA isoform pattern.

A straightforward, simple, fast, and reproducible method for purification of PSA that allows isoform PSA profiling by CZE is highly desirable.

2. Materials and methods

2.1. Samples, reagents, and devices

The European standard of fPSA was supplied by Resource Technology (Salisbury, Wiltshire, UK). A seminal plasma sample from ejaculate of a healthy individual was kindly provided by Dr. Eldiberto Fernandez and Dr. Ricardo Garcia from the Ramon y Cajal Hospital (Madrid, Spain) following the guidelines of the Ethical Committee of the Hospital. Protein A purified monoclonal (clone 5G6) anti-tPSA antibody was from Antibodies on-line (Aachen, Germany). Sulphuric acid was from Panreac (Barcelona, Spain). Sodium periodate, sodium cyanoborohydride, sodium borohydride, glycine, propionic acid, urea, Tween® 20, Tris(hydroxymethyl) aminomethane (Tris), Tris–HCl, Brij35®, sodium tetraborate, decamethonium bromide, and protease inhibitor cocktail were from Sigma–Aldrich (Steinheim, Germany). Glycerol was from VWR (Barcelona, Spain). Sodium azide was from J.T. Baker (London, UK). Disodium hydrogen phosphate, sodium dihydrogen phosphate, sodium chloride, potassium chloride, magnesium chloride, and acetonitrile were from Merck (Darmstadt, Germany). Phosphate buffered saline (PBS) consisted on 0.1 M disodium hydrogen phosphate/sodium dihydrogen