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Glycoform analysis of alpha$_1$-acid glycoprotein by capillary electrophoresis☆

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ABSTRACT

A relatively fast and reproducible CE separation was developed for the glycoform analysis of α_1-acid glycoprotein (AGP). Factors that were considered included the pH for this separation and various techniques for coating the capillary and/or to minimize electroosmotic flow and protein adsorption. Optimum resolution of the AGP glycoforms was obtained at pH 4.2 with a running buffer containing 0.1% Brij 35 and by using static and dynamic coatings of PEO on the capillary. These conditions made it possible to separate nine AGP glycoform bands in about 20 min. The limit of detection (based on absorbance measurements) ranged from 0.09 to 0.38 μM for these AGP glycoform bands, and the linear range extended up to a total AGP concentration of at least 240 μM. The migration times for the glycoform bands had typical within-day and day-to-day precisions of ±0.16–0.23% or less, respectively, on a single treated capillary and the variation between capillaries was ±0.56% or less. A charge ladder approach was employed to examine the mass or charge differences in the glycoforms that made up these bands, giving a good fit to a model in which the neighboring bands differed by one charge (e.g., from a sialic acid residue) and had an average mass difference of approximately 0.7–0.9 kDa. The approaches used to develop this separation method are not limited to AGP but could be extended to the analysis of other glycoproteins by CE.

1. Introduction

Alpha$_1$-acid glycoprotein (AGP) is an acute phase glycoprotein and serum transport protein with a low isoelectric point (pI, 2.8–3.8) and a high carbohydrate content (40%) [1,2]. The normal concentration of this protein in human plasma is 0.5–1.0 g/L (or 12–24 μM); however, this concentration can increase by up to ten-fold in some diseases [1]. In theory, more than 10^5 glycoforms of AGP could exist as a result of the five glycosylation sites that are present on this protein; however, it has been stated that only 12–20 major glycoforms of AGP are typically detected in human serum [3]. The use of AGP glycoforms as biomarkers has been explored for the diagnosis of conditions such as ovarian cancer, leukemia and atherothrombosis [4,5]. Changes in the glycoforms of AGP have also been of interest in terms of how these variations may alter the binding of this protein to drugs [1–3,6].

Capillary electrophoresis (CE) is one technique that has been used to examine AGP and its glycoforms [4,5,7–12]. The potential advantages of this approach include its efficiency, fast analysis times and small sample requirements [13]. However, interactions with the capillary wall can present a challenge in the use of CE for AGP, glycoproteins and related biomolecules [14]. The presence of such interactions can lead to low separation efficiencies and alter the electroosmotic flow, which can also change the observed mobilities over time and result in poor precision or non-reproducible separations [14,15].

To overcome these issues, some previous studies have used capillaries that contained a cross-linked coating of dimethylpolysiloxane and a dynamic coating of (hydroxypropyl)methylcellulose or buffers containing the surfactant Tween 20 for the analysis of AGP glycoforms by CE [7,8,12]. The adsorption of AGP during CE has also been reduced by using capillaries that were covalently modified with succinyl-poly-L-lysine or by using a commercial capillary with a hydrophilic bonded phase (i.e., CElect-P1) [9]. Some limitations of these previous methods have been that they either involved tedious methods for capillary treatment or they required relatively expensive pre-treated capillaries [7–9,12]. Another approach has been to employ buffer additives such as putrescene or urea to disrupt interactions between AGP and the capillary wall and to reduce electroosmotic flow [4,5,10,11]. However, the migration times of AGP glycoforms cannot be easily used to make accurate peak assignments in such methods due to the variability in these values, the relatively large number of peaks that are present, and the similar migration times of the glycoform bands [4]. Thus, there is still a

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need for a simple and reproducible CE method for examining AGP glycoforms.

In this study, a relatively fast and reproducible CE method will be optimized and developed for analyzing the glycoform pattern of AGP. The effect of pH on the resolution of the glycoform pattern will be considered, as well as the use of capillaries that are modified with permanent coatings, static coatings or dynamic coatings based on various protein-repellent polymers. The use of other additives in the running buffer will also be examined to further improve the separation of AGP glycoforms. The final CE method will be evaluated with standard samples of human AGP to determine this technique's resolution, speed, response, and precision. In addition, the results of this method will be analyzed with a charge ladder model to determine the general types of structural differences that are present between the AGP glycoforms that are present in each observed band.

2. Theory

Charge ladders have been used in the past with CE to characterize a series of related proteins [16,17]. In this current study, the electrophoretic mobility (μ_n) of a given AGP glycoform band (i.e., band number "n" in a series of bands) was described by using the following expression,

$$\mu_n = C_p \frac{z}{M^{\alpha}} \quad (1)$$

where z represents the charge of the species within the band, M is the average molar mass of these species, and α is an empirical parameter that is related to the shape and degree of solvation of the species (i.e., where α is generally believed to equal 2/3 for globular proteins) [16–19]. The term C_p in Eq. (1) is a constant that is related to the shape and solvation state of the glycoform species, as well as the ionic strength and viscosity of the running buffer [16,17]. The charge z in Eq. (1) can also be described by Eq. (2),

$$z = z_0 + nz_{seq} \quad (2)$$

where z0 represents the charge of the AGP glycoform band with the lowest charge in a series of bands, and zseq is the charge difference between neighboring bands. Combining Eqs. (1) and (2) with a value for α of 2/3 gives the equivalent expressions in Eq. (3).

$$\mu_n = \frac{C_p}{M^{2/3}}\left(z_0 + nz_{seq}\right) = \mu_0 + \frac{C_p}{M^{2/3}} nz_{seq} \quad (3)$$

Over a small range of mass, such as represented by the product of the terms n and m, the electrophoretic mobility of an AGP glycoform band will be approximately proportional to 1M (Note: see the Supplementary materials, which demonstrate this concept using data from Ref. [18]). In addition, the average molar mass of the species in an AGP glycoform band can be represented by Eq. (4),

$$M = M0 + nm \quad (4)$$

where M0 is the average mass of the AGP glycoform species in the band with the lowest charge, and m is the unit change in mass between the glycoform bands. When these expressions are placed into Eq. (3), the electrophoretic mobilities of the AGP glycoform bands can be described by the following approximate relationships.

$$\mu_n = \frac{C_p}{M_0 + nm}\left(z_0 + nz_{seq}\right) \cong \mu_0 + \frac{C_p}{M_0 + nm} nz_{seq} \quad (5)$$

For the expressions shown in Eq. (5), the product "nm" (i.e., which represents the change in mass between bands 0 and n) will often be quite small when compared to the overall and average mass M_0 for the species with the lowest charge. This situation would be expected when the value of n is relatively low (e.g., nm was less than 10% of the value for M_0 in this study when n was less than or equal to 6). This condition allows Eq. (5) to be simplified and rearranged into the form that is shown below.

$$\frac{1}{\mu_n - \mu_0} = \frac{M_0}{z_{seq} C_p}\frac{1}{n} + \frac{m}{z_{seq} C_p} \quad (6)$$

According to Eq. (6), a plot of $\frac{1}{\mu_n - \mu_0}$ versus $\frac{1}{n}$ should result in a linear relationship for such a system (Note: in this study, the value of n was found by using the absolute difference in the migration order between a given AGP glycoform band and the glycoform band with the lowest charge). The ratio of the unit change in mass versus the overall mass, m/M_0, can then be obtained by dividing the intercept by the slope for the resulting best-fit line. If M_0 is also known, the value of m can be obtained by using the relationship m = (Intercept/Slope) M_0.

3. Materials and methods

3.1. Reagents

The AGP (from pooled human plasma, ≥99% pure; catalog numbers, SLBJ6840 V and SLBG6410V), poly(vinyl alcohol) (PVA, hydrolyzed; weight-average molar mass, 31–50 kDa; 98–99%), poly(ethylene oxide) (PEO; viscosity-average molar mass, 8000 kDa), dextran (weight-average molar mass, 282 kDa), Brij 35 (number-average molar mass, 1.198 kDa) and thiourea were purchased from Sigma-Aldrich (St. Louis, MO, USA). All aqueous solutions and samples were prepared using water obtained from a Milli-Q Advantage A10 water purification system (EMD Millipore Corporation, Billerica, MA, USA).

3.2. Apparatus

The electrophoretic separations were carried out using a P/ACE MDQ instrument (Beckman Instruments, Fullerton, CA, USA). The capillary in this system was maintained at 25 °C during the separation, which was performed in most experiments at an applied potential of −30 kV (i.e., in the reversed polarity mode). This system used 60.2 cm × 50 μm I.D. fused silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) with an effective length to the detector of 50 cm. Absorbance detection was carried out at 200 nm, and the CE data were collected by using 32 Karat software 7.0 from Beckman. These data were analyzed by utilizing Peakfit 4.12 software (Systat Software, San Jose, CA, USA).

3.3. Capillary modification

Each capillary was activated by rinsing it for 30 min with 1.0 M sodium hydroxide and washing it with water for 10 min prior to use. The method that was used for the permanent capillary modification with PVA was the same as described previously [20]. The approach employed for placing a static coating of PVA, PEO or dextran on a capillary was also carried out according to a previous method [21] but used an automated rinsing protocol. In the static coating method, the capillary was cleaned for 5 min with 1.0 M sodium hydroxide, which was followed by a 3 min wash with water. The coating was then applied by using a 5 min rinse of 1.0 M hydrochloric acid and a 5 min rinse with a 0.2% (w/v) polymer solution that contained PEO, PVA or dextran along with 0.10 M hydrochloric acid. Application of the polymer solution was followed by a 5 min rinse with the running buffer that was to be used in the CE method. All rinses were performed at an applied pressure of 50 psi in the forward direction. A 0.05% (w/v) solution of the desired polymer (e.g., PVA, PEO, or dextran) was dissolved into the running buffer to create a dynamic coating during the CE separation.