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Selective separation of ferric and non-ferric forms of human transferrin by capillary micellar electrokinetic chromatography

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ABSTRACT

The previously published method allowing the separation of non-ferric (iron-free) and ferric (iron-saturated) forms of human serum transferrin via capillary electrophoresis has been further developed. Using a surface response methodology and a three-factorial Doehlert design we have established a new optimized running buffer composition: 50 mM Tris–HCl, pH 8.5, 22.5% (v/v) methanol, 17.5 mM SDS. As a result, two previously unobserved monoferric forms of protein have been separated and identified, moreover, the loss of ferric ions from transferrin during electrophoretic separation has been considerably reduced by methanol, and the method selectivity has been yet increased resulting in a total separation of proteins exerting only subtle or none difference in mass-to-charge ratio. The new method has allowed us to monitor the gradual iron saturation of transferrin by mixing the iron-free form of protein with the buffers with different concentrations of ferric ions. It revealed continuously changing contribution of monoferric forms, characterized by different affinities of two existing iron binding sites on N- and C-lobes of protein, respectively. Afterwards, the similar experiment has been conducted on-line, i.e. inside the capillary, comparing the effectiveness of two possible modes of the reactant zones mixing: diffusion mediated and electrophoretically mediated ones. Finally, the total time of separation has been decreased down to 4 min, taking the advantage from a short-end injection strategy and maintaining excellent selectivity.

1. Introduction

Human serum transferrin (Tf) is the iron-binding transport protein essential for delivering of iron ions into the cells. It binds two trivalent iron (ferric) ions to the N- and C-binding sites, respectively, resulting in the entirely iron-saturated form of protein (holo, h-Tf). Alternatively only one ferric ion can be bound to the N- or C-binding site, hence two possible monoferric forms of Tf may exist: Fe_N–Tf and Fe_C–Tf [1,2]. The efficient separation of iron-free (apo, a-Tf), monoferric, and holo forms was a problematic task, because they exhibit the structural and chemical similarity [3].

Both a capillary zone electrophoresis and a micellar electrokinetic chromatography (MEKC) have been reported to be suitable techniques for the analysis of proteins and peptides [4–6]. There are numerous parameters which may be adjusted to improve the separation, however, the fast method optimization is concurrently impeded. Therefore, the use of experimental design approach is advisable, especially in the case when the results seem to be dependent on several factors with no determined correlation.

MEKC, due to the surfactant presence, minimizes the adsorption of proteins to the capillary inner surface, thus making the results more precise and reproducible [7–10]. Tf was a subject of several CE-based analysis conducted usually with the use of MEKC. These studies were mainly focused on its determination in the biological material, including determination of a carbohydrate-deficient transferrin, a common marker of alcohol abuse [11–15]. Recently, we have also published a method allowing the separation of a-Tf and h-Tf using MEKC technique [16]. Our protocol was based on the addition of 7 mM SDS to running buffer consisting of 50 mM Tris–HCl, pH 8.5. Unfortunately, the presence of the two monoferric forms of Tf (Fe_N–Tf and Fe_C–Tf) could not be confirmed by using described method.

Our present work was aimed at the further method development to enable the detection of all four forms of Tf including the differentiation between Fe_N–Tf and Fe_C–Tf, prevention from iron release during electrophoretic separation, and reduction in

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separation time. A particular effort has been made to provide the evidence that the peaks observed on the electropherograms are the assumed Tf forms, and to investigate the contribution of each ferric form at different saturation levels. Distinct analytical purpose was to attempt two modes of on-line protein saturation conducted directly inside the capillary, and to use the experimental design and the surface response methodology for method optimization.

2. Materials and methods

Human a-Tf, human h-Tf (powder, BioReagent, suitable for cell culture, ≥98%), and human serum albumin (HSA) (powder, fatty acids free, globulin free, ≥99%), iron(III) nitrate nonahydrate, and nitrilotriacetic acid (NTA) were obtained from Sigma–Aldrich (Germany). Chemicals of analytical reagent grade: sodium hydroxide and ethanol were supplied by POCH S.A. (Gliwice, Poland), while LC–MS grade solvents: methanol, isopropanol an acetonitrile were purchased from Sigma–Aldrich (Germany).

All solutions were prepared in MiliQ quality water and filtered through 45 μm regenerated cellulose membrane and degassed by centrifugation. All separation buffers were stored at +4 °C, while NaOH and HCl solutions at ambient temperature. The samples of proteins were prepared in 50 mM Tris buffer, pH 8.5 with neither surfactant nor organic solvent addition, at the final concentration ranging from 0.10 to 0.50 mg/mL. The accurate value of buffer pH was obtained by using concentrated HCl (Tris–HCl). Before each analysis, samples were centrifuged for 5 min, 5000 × *g*. The minimal volume of the sample was 50 μL.

The measurements were performed using a P/ACE Capillary Electrophoresis System (Beckman-Coulter, USA) equipped with a diode array detector. During all experiments the whole spectra within the range of 200–600 nm were collected, however, the results obtained at 200 nm were used in further processing. The uncoated fused-silica capillaries (laser burned detection window) were of 60 cm × 50 μm i.d. or 30 cm × 50 μm (Beckman-Coulter), with a 50 or 20 cm distance to the detector, respectively. Temperature of the sample tray and capillary were set up to 22 °C. The capillary was being rinsed between runs as follows: 0.138 MPa (20 psi) of MiliQ water for 2 min, 0.138 MPa of 0.1 M NaOH for 3 min; and 0.138 MPa of running buffer for 3 min. Before the first run at a working day the rinsing protocol involved 0.138 MPa of methanol for 6 min; 0.138 MPa of 0.1 M HCl for 4 min; 0.138 MPa of MiliQ water for 3 min; 0.138 MPa of 0.1 M NaOH for 10 min; and 0.138 MPa of running buffer for 10 min, whereas in case of the first use of the capillary after mounting in cartridge the same procedure has been used, but the duration of all steps was doubled. Sample injection was performed using a forward pressure at anodic side, applying: 3.4 kPa (0.5 psi) for 5 s, unless stated otherwise. Forward voltage ranging from 15 kV to 30 kV was being applied. Each analysis has been repeated at least three times. The instrumental noise produced during detection has been smoothed out using Origin 8.0 software (OriginLab Corporation, USA). During chemometric calculations Statistica 10 (StatSoft Inc., Tulsa, OK, USA) software was used.

Conductivity measurements were performed using a microcomputer conductivity meter (Elmetron CC-551) with a conductivity sensor (CD-2 type, 0.51 cm^{-1} sensor constant).

3. Results and discussion

3.1. Addition of organic solvent

In the beginning, the effect of four different organic solvents as the buffer additives has been tested: methanol, ethanol, propan-2-ol, and acetonitrile, each one in final 20% (v/v) concentration. The solvents were added to the 50 mM Tris buffer, pH 8.5, containing 10 mM SDS. According to the previously reported data, in this buffer SDS is able to form the micelles, probably a crucial factor enabling the separation of holo and apo forms of Tf. A reference buffer was the buffer without the addition of any organic modifier. The results have been generally compared by separating the sample containing mixed h-Tf, a-Tf and HSA. HSA was used as an internal standard to compare the obtained relative migration times standing for the particular transferrin forms and for calculation of relative peak areas.

Among the four tested organic solvents, each one resulted in altered electropherogram, however only in the case of methanol the appearance of the two minor, totally separated peaks has been reported, localized in a gap between the peaks corresponding to h-Tf and a-Tf. Another effect which has been observed for methanol was the change in peak intensity, i.e. the peak standing for h-Tf had similar intensity as that for a-Tf, contrary to the buffer without methanol where the peak of h-Tf was considerably diminished. It has supported us to conclude, that the loss of iron by h-Tf can be minimized in presence of the methanol (see Supplementary Material for more detailed investigations of the role played by methanol).

Apart from the organic solvents, also the addition of urea in final concentration of 6 M, 1 M, and 1 M with combination with 20% methanol, has been investigated. Urea was reported to improve separation of Tf forms according to the iron saturation in gel electrophoresis [17]. In our case it has turn out, that the changes in electropherograms have been more extensive than those caused by the solvents, however the peaks were incompletely separated and vastly diminished, i.e. the sensitivity of the method was considerably weaker after addition of urea. In the end, we have concluded that methanol without urea is the most promising buffer additive.

Then, the effect of increasing concentration of methanol in the running buffer has been investigated, as it has been shown in Fig. 1. Final concentration of Tris 50 mM, SDS 10 mM and pH 8.5 were kept for each buffer. The methanol-free buffer and the buffers containing: 10.0%, 17.5% and 25.0% (v/v) methanol were compared. It has revealed the strong and increasing with concentration effect of this additive on the peak area ratio between holo and apo forms, and the appearance of two novel peaks. We have hypothesized, that these peaks may originate from monoferric forms of protein, previously unobserved. The analysis performed for the pure h-Tf and a-Tf resulted also in the appearance of these peaks, but their intensity was very low.

3.2. Experimental design

Taking into account that finding of the optimal conditions for a method involving addition of the two different but crucial buffer components is not a trivial task, we have decided to use experimental design approach. For that purpose, set of experiments based on a three-factorial Doehlert uniform shell design and surface response methodology was conducted [18]. The optimized factors were: concentration of methanol c_{MeOH}, concentration of SDS c_{SDS} and pH. Preliminary experiments performed initially helped us to point out the range of optimized factors (see Supplementary Material for more details) [19]. Three system responses have been chosen: time of analysis defined as time of the end of HSA peak, sum of FWHM (Full Width at Half Maximum) for all five peaks of interest, and inverse resolution between h-Tf and HSA peaks. Such defined responses reflect the improvement in separation if their values are decreasing. According to the surface response methodology, a quadratic polynomial response model for the sample was