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Separation of iron-free and iron-saturated forms of transferrin and lactoferrin via capillary electrophoresis performed in fused-silica and neutral capillaries

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ABSTRACT

A capillary electrophoresis-based method for the cost-effective and high efficient separation of iron-free and iron-saturated forms of two members of transferrin family: transferrin and lactoferrin has been developed. The proposed qualitative method relying on the SDS application allowed us to separate iron-free and iron-saturated forms of these proteins, as well as human serum albumin, used as an internal standard. Owing to the distinct migration times under established conditions, the combination of transferrin and lactoferrin assays within a single analytical procedure was feasible. The performance of the method using a fused-silica capillary has been compared with the results obtained using the same method but performed with the use of a neutral capillary of the same dimensions. Neutral capillary has been used as an alternative, since the comparable resolution has been achieved with a concomitant reduction of the electroosmotic flow. Despite of this fact, the migration of analytes occurred with similar velocity but in opposite order, due to the reverse polarity application. A quantitative method employing fused-silica capillary for iron saturation study has been also developed, to evaluate the iron saturation in commercial preparations of lactoferrin.

1. Introduction

The aim of this work was to develop a qualitative method enabling the separation of iron-free and iron-saturated forms of transferrin (Tf) and lactoferrin (Lf).

Transferrin is a serum glycoprotein responsible for transportation of iron. Depending on the number of trivalent iron ions bounded to the protein, one can distinguish: free-iron form, i.e. apo-transferrin (a-Tf), two distinct monoferric-transferrins having iron bounded at C- or N-terminal binding site, respectively, and differic iron-saturated form, i.e. holo-transferrin (h-Tf). Lactoferrin is a member of the same protein group, and analogously to Tf may exist in apo- and holo-forms. Lf is present in other extracellular excretions, i.e. in milk, saliva or tears [1–3]. Lf is believed to be one of the components of the immune system of the human body because of its antimicrobial activity. Influence of Lf on bacterial, viral, protozoan, and fungal pathogen growth has been numerously investigated [4–6]. Additionally Lf has been reported to be a part of innate defence [7,8], therefore, there is a current interest in additional its supplementation [9–11].

The insight into iron saturation level in case of these proteins has another biomedical relevance. Tf is a potential carrier protein for numerous drugs, from which a part may interact with a-Tf and h-Tf in different manner or with different strength [12–17]. The metabolism of iron is also related to a range of disorders affecting humans, therefore their diagnosis might be facilitated by the knowledge about iron saturation of Tf [18,19]. Antimicrobial effects of Lf may in turn be different for a-Lf and h-Lf. The former one has already been described as more potent in reducing the growth of some bacterial strains, such as *Staphylococcus aureus* and *Escherichia coli* [20,21]. Only a high performance analytical technique may guarantee the efficient separation of these molecular forms due to its chemical and structural similarity.

Among the numerous instrumental techniques of protein separation, capillary electrophoresis (CE) is characterised by the high efficiency, small amounts of sample, short analysis time and significant automation of the assay [22]. One of the largest practical obstacles encountered during method development is a tendency of protein to be adsorbed onto the negatively charged inner surface of a capillary wall. It results in peak broadening, disturbance of

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electroosmotic flow (EOF) and decrease of capillary lifespan. Several types of reagents were proved to be effective in solving this problem, e.g. semi-permanent and dynamic coating materials and, to a lesser extent, detergents used only as the buffer additives. As far as the use of advanced coating is burdened with high costs of reagents, various detergents are present virtually in all chemical laboratories. Another possibility to reduce protein adsorption is the use of neutral capillary, chemically different, devoid of charge on its inner surface. Neutral capillary eliminates EOF, thus upon the voltage application only one type of ions starts to migrate towards electrode, unless additional pressure is applied. While a vast majority of analysis is being performed using fused-silica capillaries, the implementation of neutral ones is restricted only to few examples in the literature [23]. One of the reasons for that is several times higher cost of the neutral capillary.

To our knowledge, there is no described CE-based protocol enabling the totally resolved separation of iron-free (apo) and iron-saturated (holo) forms either for Tf, or Lf. There was the reported procedure enabling the separation of Tf and albumin from urine samples by MEKC, but the method was not selective regarding apo- and holo-forms [24,25]. Still, gel isoelectric focusing was reported to be useful to separate monoferric and differic forms of Tf, revealing their isoelectric points to be 5.6 and 5.2, respectively [26]. To estimate the iron saturation level, other analytical techniques were applied, as spectrophotometry, ICP-MS, ELISA, fast protein liquid chromatography, often in their combination [27–29].

In this work authors would like to demonstrate the analytical potential of the surfactant enhanced capillary electrophoresis technique, which has been employed to separate apo- and holo-forms in samples containing Tf, Lf, or both proteins within a single run. Hence, the comparative studies of Tf and Lf in the function of their iron saturation level can be combined within one method.

2. Materials and methods

2.1. Chemicals

Human apo-transferrin (powder, BioReagent, suitable for cell culture, ≥98%), human holo-transferrin, albumin from human serum (HSA) (powder, fatty acids free, globulin free, ≥99%) was obtained from Sigma–Aldrich (Germany). Bovine lactoferrin was purchased from DMV International (estimated purity, 95%; Veghel, the Netherlands). Apo- and holo-lactoferrin were prepared on site as previously described with estimated purity of 98 and 80%, respectively [27]. All chemicals were of analytical reagent grade: sodium hydroxide, citric acid and sodium chloride supplied by POCH S.A. (Gliwice, Poland) and other chemicals were purchased from Sigma–Aldrich Co.

All solutions have been prepared in doubly distilled water and filtered through 45 μm regenerated cellulose membrane and degassed by centrifugation. All buffers were stored at +4 °C, while NaOH and HCl solutions at ambient temperature.

2.2. Samples

The samples of proteins were prepared in 20 mM Tris buffer, pH 8.5 with no surfactant addition, at the final concentration 0.2 mg/mL. Before each analysis, the solutions were centrifuged for 5 min, 20,000 RCF. The minimal volume of the sample was 50 μL. HSA was used as an internal standard to calculate the relative migration times and peak heights.

2.3. Instrumentation

The measurements were performed using a P/ACE Capillary Electrophoresis System (Beckman-Coulter, USA) equipped with a diode array detector. During all experiments the detection was performed at analytical wavelength of 200 nm. Both an uncoated fused-silica capillary, and neutral (polyacrylamide covalently attached to inner wall, laser burned detection window) capillary were of 60 cm × 50 μm i.d. (Beckman-Coulter), with a 50 cm distance to the detector. Temperature of the sample tray and capillary were set up to 22 °C. The rinsing procedure between runs included following steps: 20 psi of methanol for 3 min; 20 psi of 1 M HCl for 2 min; 20 psi of distilled water for 1 min; 20 psi of 0.1 M NaOH for 3 min; and 20 psi of running buffer for 5 min. The rinsing procedure of neutral capillary was: 20 psi of distilled water for 3 min; 20 psi of 0.1 M HCl for 3 min; and 20 psi of running buffer for 3 min. For the both type of capillaries, before the first run at a working day, the time of every step was doubled, and in case of the first use of the capillary after mounting in cartridge, it was extended four times. Before the installing, neutral capillary was stored at +4 °C, afterwards in instrument with the ends dipped into distilled water.

Sample injection was conducted using a forward pressure at anodic side for fused-silica capillary, and cathodic side for neutral capillary applying: 0.5 psi for 5 s. Forward voltage: 15 kV, 20 kV or 30 kV (ramp time: 0.17 min) was applied during analysis performed with fused-silica capillary, whereas neutral one required application of reverse polarity with maintenance of the same absolute voltage value. The measurements were repeated at least three times, however five repetitions were done if the data proceeded statistical analysis.

The instrumental noise produced during detection has been smoothed out using Origin 8.0 software (OriginLab Corporation, USA).

3. Results and discussion

3.1. Method optimisation

3.1.1. Effect of detergent

The first analysis was performed for the mixture of h-Tf, a-Tf, h-Lf, a-Lf and HSA, using the fused-silica capillary, 20 kV voltage in normal polarity, and 50 mM Tris, pH 8.0 as the back-ground electrolyte (BGE). As it can be seen in Fig. 1-S in supplementary materials, it resulted in the appearance of only two well-separated peaks. As it has been revealed afterwards, they stood for total Tf and HSA, respectively. Lf has not been detected even if additional forward pressure of 0.5 psi was applied and data collection extended to 30 min. This could derive from the extensive adsorption of Lf molecules to the inner surface of capillary. It is worth mentioning, that at pH 8.0 unlike to Tf and HSA, Lf was positively charged, since its pI was suggested to be about 8.7. As a result, Lf molecules could strongly interact with negatively charged silanol groups by Coulomb's force.

Hence, the separation of apo- and holo-forms of proteins of interest was stated to be unfeasible under proposed conditions. As a potential solution of the problem, the searching for the proper detergent used as a buffer additive has been postulated. Among them: Tritone X-100, lauryldimethylamine *N*-oxide (LDAO), and sodium dodecyl sulphate (SDS) have been tested, each one in concentration 0.2% (w/v). Tritone X-100 did not give any improvement, while LDAO resulted merely in a shorter peak widths. Only the addition of SDS has extremely improved the resolution, revealing the five, nearly entirely separated peaks (see Fig. 1-S.B). These five forms might be successfully separated probably due to their interactions with negatively charged SDS molecules. Various affinity of an individual kind of protein molecules to hydrophobic tails or polar heads of surfactant molecules arranged in the specific micelles