SEPARATION OPTIMIZATION OF THE MOLECULAR FORMS OF TRANSFERRIN COMPLEXES BY CAPILLARY ZONE ELECTROHORESIS

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Glicoproteina transferina serică umană (Tf) are două loburi. Fiecare lob are un situs de legare a ionilor de fier trivalent. În urma legării ionilor de metale şi a diferitelor anioni (oxalat, aziridin-carboxilat, bicarbonat) la acest situs se pot obţine patru forme moleculare a transferinei (holo-Tf, diferri Fe_NTfFe_C, şi monoferri $TfFe_N$, $TfFe_C$). Experimete de electroforeză capilară de înaltă performanţă (CZE) a fost utilizate pentru a separa aceste forme moleculare. Pentru a obţine o separere de înaltă eficacitate a diferitelor forme moleculareobţinute în urma reacţiei de complexare am schimbat următoarele condiţii de separare: electrolitul, tratarea capilarelor, tensiunea.

Human serum transferrin (Tf), a glycoprotein, has two lobes, each having a binding site for trivalent iron ions. The binding of metal ions and different anions (oxalate, aziridine-carboxylate, bicarbonate) to these binding site results four different molecular form of Tf (holo-Tf, diferri Fe_NTfFe_C, and monoferri $TfFe_N$, $TfFe_C$). High performance capillary zone electrophoresis (CZE) experiments were performed in order to separate these molecular forms. We changed the following separation conditions: background electrolyte, capillary treatment and voltage to achieve high efficiency of separation of transferrin probes after the complexation reaction.

Keywords: transferrin, capillary zone electrophoresis

1. Introduction

Human serum transferrin (Tf) is an iron-transport glycoprotein having a bilobal structure folded into two structurally homologous globular lobes referred

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to as the N and C lobes, with very similar folds and essentially identical iron binding sites. The iron binding site consist of four protein ligands (2 Tyr, 1 His and 1 Asp) plus two oxygen atoms from the synergistically bound CO_3^{2-} anion [1, 2]. In *vitro* this synergistic anion is replaceable with some other anions, which play a role at the formation of a stable bond between trivalent iron ions and Tf [3-5]. Depending on the number of bounded trivalent iron ions different molecular forms of human serum transferrin are obtained. The four molecular forms of transferrin, i. e. iron-free or apotransferrin (apo-Tf), two types of monoferric-transferrins ($TfFe_N$ and $TfFe_C$) having iron bound at the N- or C-terminal binding site, respectively, and the diferric-transferrin (FeNTfFeC) differ only in the number of bounded ferric ions and synergistic anions. These large molecular analogies require high performance techniques to separate them. Capillary zone electrophoresis (CZE) is a convenient technique to separate big protein molecules with high efficiency [6].

Our aim is to develop the conditions for better separation of the different molecular forms of transferrin-complexes (realised using different synergistic anions e. g. bicarbonate, oxalate and aziridine-carboxylate) by CZE technique. In this paper we summarize the results obtained using different separation condition and analyze the effectiveness of the different applied conditions, determining the optimal technical conditions.

1. Experimental part

1.1 Materials and methods

1.1.1 Reagents and apparatus

All used reagents were of analytical grade. Iron-saturated human serum transferrin was purchased from Sigma-Aldrich, the iron-free transferrin from Behring Werke AG (Marburg, Germany). Sodium hydrogen carbonate ($NaHCO_3$) was purchased from Reanal (Budapest, Hungary), the iron(III)-chloride powder from Sigma-Aldrich, the chelating agent nitrilotriacetic acid (NTA) was a Fluka product. Other chemical reagents were purchased from Sigma-Aldrich, Reanal or Fluka. For capillary electrophoresis measurements a Biofocus 3000 (Bio-Rad) equipment was used.

1.1.2 Capillary coating

Glass capillaries with 50 μm ID were coated with non-crosslinked acrylamide to eliminate electroendosmosis, using a modified method described by Kiár et. al [7]. The capillaries were pre-treated before coating, washed with methanol, NaOH, HCl, water and were dried with air. For sylanization 50 %

solution of γ-methacryloxypropyltrimethoxysilane in 50 % acetone was sucked into the capillaries. After overnight incubation at room temperature the solution was withdrawn and the capillaries were washed with acetone and were filled with a 4 % acrylamide solution containing 5 % N,N, N', N'-tetramethylethylenediamine (TEMED) and 5 % ammonium persulfate. After 2 hours the tube was rinsed with water and dried with air.

1.1.3 Capillary zone electrophoresis

For capillary zone electrophoresis measurements a Biofocus 3000 (Bio-Rad) equipment and 300 mm effective length coated and uncoated glass capillaries with 50 μm ID were used. For these experiments we used 20 mM phosphate buffer (pH=2.5), 100 mM 2-(N-morpholino) ethanesulfonic acid (MES, pH=6,1), and a mixture of 18 mM Tris, 18 mM 2,2',2'',2'''-(ethane-1,2-diyldinitrilo) tetraacetic acid (EDTA) [6], and 0.3 mM boric acid (pH=8.4) as background electrolyte. Sample concentration varied between 5-14 mg Tf/ml. The samples were injected using 1-10 psi. For measurements 5-10 kV voltage and 20 °C were used. Uncoated capillaries were conditioned with 1 M NaOH (180 sec), 1 M HCl (180 sec), and the coated capillaries with 1 mM HNO_3 (180 sec), and 0.015 % methyl cellulose (120 sec).

1.1.4 Separation efficiencies

The efficiency of separation systems was determined by the theoretical plate numbers (N) and peak capacities (which predict that theoretically how many component can be separated per peak), were calculated using the following equations.

$$N = 16 \cdot \left(\frac{t}{w}\right)^2 \tag{1}$$

$$\mathrm{n} = 0{,}5 \cdot \sqrt{\mathrm{N_{max}}} \tag{2}$$

Where: N-plate number (dimensionless)
t-component's migration time, [s]
w-peak length
N_{max}-the maximum plate number (dimensionless)

Comparing the efficiency of different separation systems, the theoretical plate numbers are expressed in length of capillary/meter.

2. Results and discussion

2.1 Separation of transferrin-complexes with capillary zone electrophoresis technique

Capillary electrophoresis, within capillary zone electrophoresis (CZE) has become a routine method for analysis of serum proteins in many clinical laboratories also. Serum proteins were separated by CZE in coated and uncoated capillaries [8].

During the presented work iron-free and iron-saturated transferrins were analyzed in the capillary zone electrophoresis experiments using different condition to obtain high separation efficiency. Changing the separation conditions, ex. pH of background electrolyte, voltage, and the capillary treatment or capillary coating, optimization of the CZE separation was achieved.

The buffer's pH is one of the most important in proteins separations [9]. Proteins have a low net charge, which can determine the direction and the speed of migration. The proteins positive charge can interact with the negative charge of the capillary wall's silanol groups, and thus the adsorption of proteins to the capillary wall occurs. This adsorptions cause unreproductive migration time and peak asymmetry. In order to reduce the interaction between the proteins and capillary wall, we changed the convenient buffer and pH, thus eliminate the adsorption or we tried to reduce this interaction by special capillary coating.

During capillary zone electrophoresis experiments phosphate buffer (pH=2.5), 2-(N-morpholino) ethanesulfonic acid (MES, pH=6.1), and a mixture of 2-amino-2-hydroxymethyl-propane-1,3-diol (TRIS), 18 mM 2,2',2",2"'-(ethane-1,2-diyldinitrilo) tetraacetic acid (EDTA) and boric acid (pH=8.4) were used as background electrolyte.

Using low pH value (phosphate buffer, pH=2.5) at the separation of Tf-iron(III)-aziridine-carboxylate, an asymmetric shape of the peak formed. This is called the "tailing" phenomenon (Fig. 1), and is caused by the slower migration of sample's particles than the buffer's particles. This phenomenon was also observed using pH=8.4 background electrolyte (mixture of 18 mM TRIS, 18 mM boric acid and 0.3 mM EDTA) (Fig. 5).

By using MES buffer, pH=6.1, this "tailing" phenomenon was eliminated. The measurements were conducted at 8 and 10 kV respectively, both resulting one peak and a shoulder on the peak's right side (Figs. 2, 3). This shoulder formation is more accentuate on the electropherogram obtained using 8 kV (Fig. 2).

In order to obtain an accurate separation of the transferrin complexes isoforms the voltage was decreased. Between the applied voltage, conductibility and the temperature are strong connections.

Increasing the conductibility of background electrolyte is directly proportional to the temperature increasing. The current are changing as long as to achieve the steady state, and the standard temperature gradient is not possible to obtain using constant voltage during the separation.

The generated Joule heating eliminates through the capillary wall. Thermal effects are irrelevant until it is within the Ohm's law availability. Based on these conditions (temperature, buffer's pH) and the capillaries inner diameter, the maximum usable voltage can be determined. In the case of capillaries with 50 µm inner diameter the maximum voltage is 20 kV [9]. In the presented experiments 5-10 kV were used. The effect of the applied constant voltage on the separation's effectiveness is presented in the Fig. 8, 9.

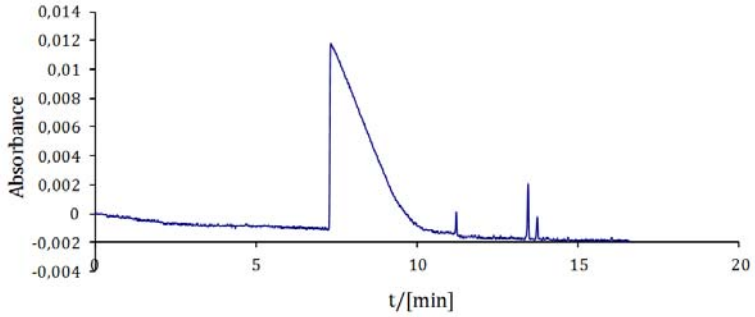

Fig. 1. Capillary zone electrophoresis of the Tf-iron(III)-aziridine-carboxylate complex.

The experimental conditions in fig. 1 have been: sample concentration 14 mg/ml Tf-iron(III)-aziridine-carboxylate complex, uncoated glass capillary, capillary dimension: 50 µm ID and 285 mm effective length, 10 psi sample injection, 20 mM phosphate buffer (pH 2,5) as background electrolyte, voltage 8 kV, current 19,4 µA, 20°C, on-tube detection at 280 nm.

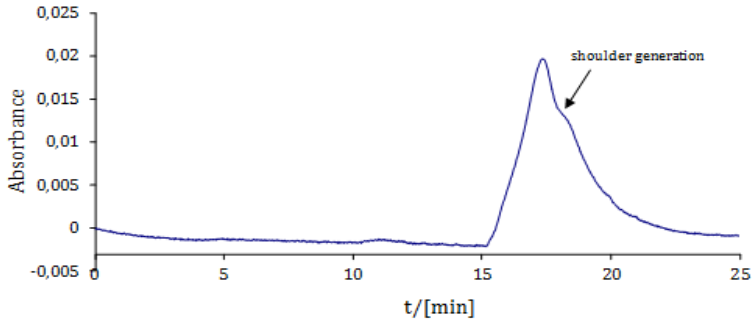

Fig. 2. Capillary zone electrophoresis of the Tf-iron(III)-aziridine-carboxylate complex

The experimental conditions in fig. 2 have been: sample concentration 14 mg/ml Tf-iron(III)-aziridine-carboxylate complex, uncoated capillary, capillary

dimension: 50 µm ID and 285 mm effective length, 10 psi sample injection, background electrolyte 100 mM MES buffer (pH 6,1), voltage 8 kV, current 18-20 µA, 20°C, on-tube detection at 280 nm.

Using 5 kV voltages we managed to obtain two distinct peaks (Fig. 4) and a shoulder on the left side of the peak 2. Based on the molecular mass of the Tf isoforms the first peak represents the diferri-Tf-iron(III)-aziridine-carboxylate complex, the second peak represents the mixture of monoferri- and iron-free form of Tf-complexes and the shoulder could predict the presence of the iron-free form of Tf in the sample. The pH of the buffers, used as background electrolytes are in strong connection with the pI of the protein samples. The isoelectric point range of transferrin's molecular form is 5.25-6.3 [10]. These could explain the high separation efficiency of the transferrin sample's, using 100 mM MES buffer (pH=6.1) as background electrolyte.

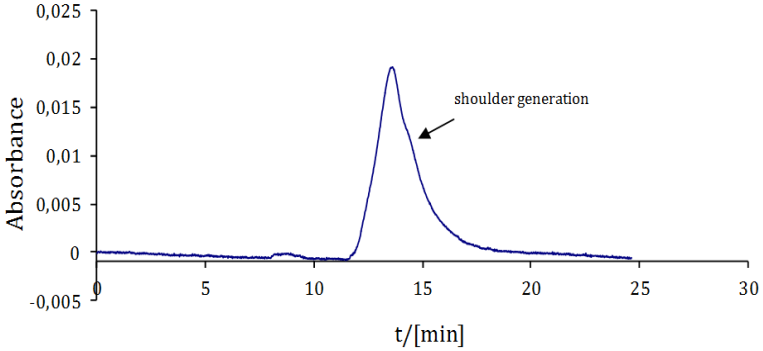

Fig. 3. Capillary zone electrophoresis of the Tf-iron(III)-aziridine-carboxylate complex

The experimental conditions in fig. 3 have been: sample concentration 7 mg/ml Tf-iron(III)-aziridine-carboxylate complex, uncoated capillary, capillary dimension: 50 µm ID and 285 mm effective length, 10 psi sample injection, background electrolyte 100 mM MES buffer (pH 6,1), voltage 10 kV, current 18-20 µA, 20°C, on-tube detection at 280 nm.

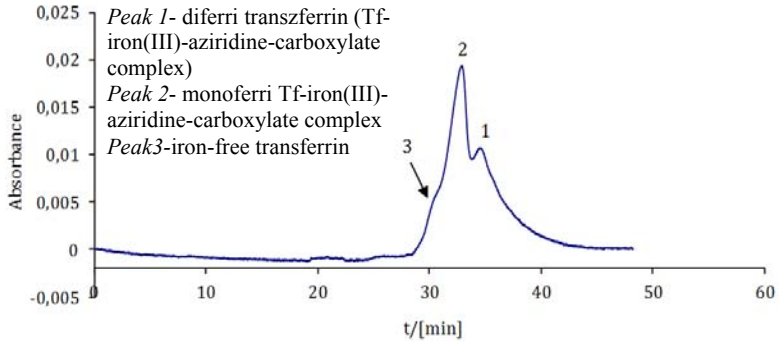

Fig. 4. Capillary zone electrophoresis of the Tf-iron(III)-aziridine-carboxylate complex

The experimental conditions in fig. 4 have been: sample concentration 7 mg/ml Tf-iron(III)-aziridine-carboxylate complex, uncoated capillary, capillary dimension:, 50 μm ID and 285 mm effective length, 10 psi sample injection, background electrolyte 100 mM MES (pH 6,1), voltage 5 kV, current 18-20 μA, 20°C, on-tube detection at 280 nm.

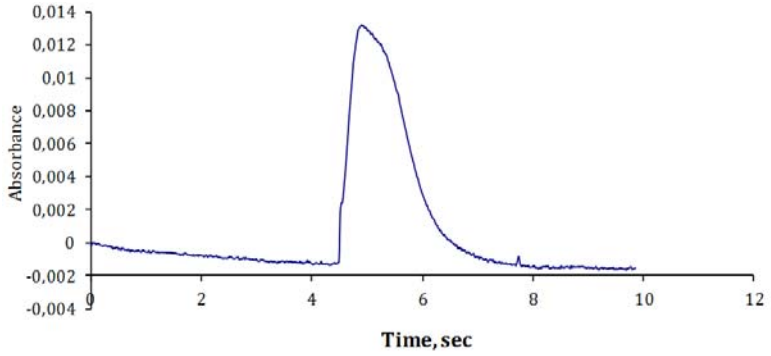

Fig. 5. Capillary zone electrophoresis of the Tf-iron(III)-aziridine-carboxylate complex

The experimental conditions in fig. 5 have been: sample concentration 7 mg/ml Tf-iron(III)-aziridine-carboxylate complex, uncoated capillary, capillary dimension: 50 μm ID and 285 mm effective length, 10 psi sample injection, background electrolyte 18 mM Tris, 18 mM EDTA, 0,3 mM boric acid (pH 8,4), voltage 8 kV, current 19,4 μA, 20°C, on-tube detection at 280 nm.

Using modified or coated capillaries the electroendosmosis (EOF) and the adsorption of the protein samples to the capillary wall can be eliminated. Coating polymers act by masking the active silanol groups, but also by increasing the viscosity in the electric double layer [11]. By shielding the capillary surface covalent coating improves the separation of proteins in terms of efficiency and resolution. Eliminating of the EOF using coated capillaries we could separate the different molecular forms of Tf-iron(III)-oxalate and Tf-iron(III)-bicarbonate complexes (Figs. 6, 7) with higher efficiency.

Coating of the capillary inner surface with non-crosslinked polyacrylamide is the most common method to eliminate EOF [12]. Capillaries coated with this method are very stable in the pH range of 2.5-8.5. The use of a mixture of 18 mM TRIS, 18 mM boric acid and 0.3 mM EDTA as background electrolyte with higher pH (pH=8.4) than the pI value of the transferrin samples, we obtained a repulsive interaction between the negative charges of the capillary wall and of the proteins.

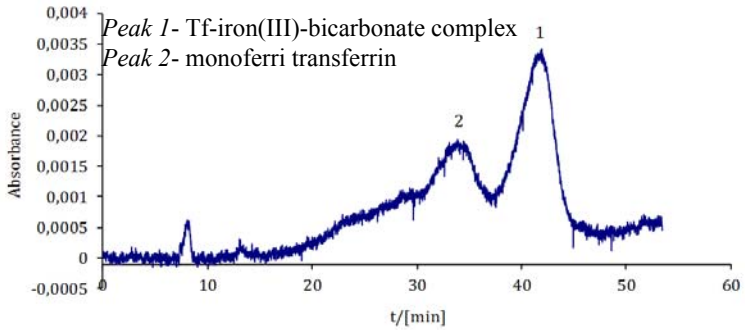

Fig. 6. Capillary zone electrophoresis of the mixture of apo-Tf and Tf-iron(III)-bicarbonate complex

The experimental conditions in fig. 6 have been: sample concentration 5 mg/ml apo-Tf and 5 mg/ml Tf-iron(III)-bicarbonate complex, glass capillary coated with non-crosslinked polyacrylamide, capillary dimension: 50 µm ID and 280 mm effective length, 5 psi sample injection, background electrolyte 18 mM Tris, 18 mM EDTA, 0.3 mM boric acid (pH 8.4), voltage 9 kV, current 15-26 µA, 20°C, on-tube detection at 280 nm.

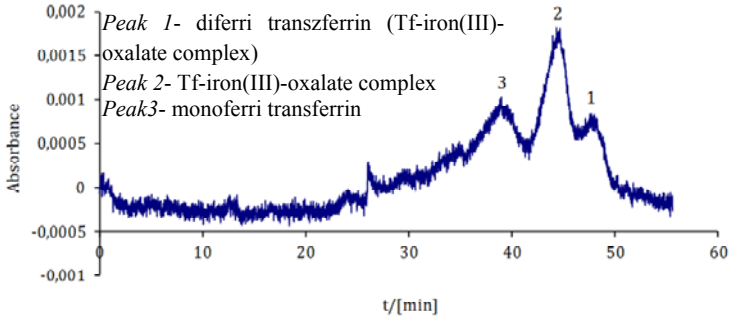

Fig. 7. Capillary zone electrophoresis of the mixture of apo-Tf and Tf-iron(III)-oxalate complex

The experimental conditions in fig. 7 have been: sample concentration 5 mg/ml apo-Tf and 5 mg/ml Tf-iron(III)-oxalate complex, glass capillary coated with non-crosslinked polyacrylamide, capillary dimension:50 µm ID, 280 mm effective length, 5 psi sample injection, background electrolyte 18 mM Tris, 18 mM EDTA, 0.3 mM boric acid (pH 8.4), voltage 9 kV, current 15-26 µA, 20°C, on-tube detection at 280 nm.

Comparing the separation using coated and uncoated capillaries at the same voltage and background electrolyte we found that eliminating of electroendosmosis (EOF) by capillary coating produce better separation (fig. 5, 6, and 7). In the case of Tf-iron(III)-bicarbonate complex we obtained only two well determined peaks (fig. 6). Supposedly the two different peaks contain the two monoferri forms of Tf-complexes. Separation of Tf-iron(III)-oxalate in the same condition proved to be more efficiency (fig. 7), it can be identified also a third peak, which supposedly determine the amount of Fe_NTf-iron(III)-oxalate and $TfFe_C$-iron(III)-oxalate complexesThe difference between the separations of two Tf-complexes based on the number of well defined peaks we explain with the efficiency of the complexation reaction. This complexation reaction can influence by the used anions (oxalate, bicarbonate or aziridine-carboxylate) and its *in vitro* affinity to the protein iron binding site.

2.2 Separation efficiencies

The effects of voltage, background electrolyte's pH, coated and uncoated capillaries on the plate number (N) (Fig. 8) and peak capacity (n_c) (Fig. 9) were investigated to optimize the separation conditions of the different molecular forms of transferrin-complexes (Tf-Fe^{3+}-oxalate, Tf-Fe^{3+}-aziridine-carboxylate and Tf-Fe^{3+}-bicarbonate).

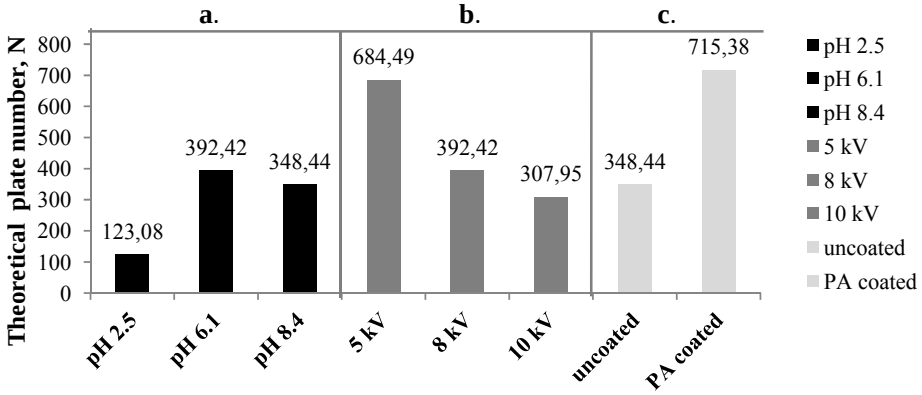

Fig. 8. Separation efficiency measured as plate numbers calculated for different background electrolyte pH, voltage, and for uncoated- and linear polyacrylamide-coated capillaries

The experimental conditions in fig. 8 have been: (a.) sample concentration 14 or 7 mg/ml Tf-Fe^{3+}-aziridine-carboxylate, background electrolytes: 20 mM PBS pH 2.5, 100 mM MES pH 6.1, 18 mM TRIS- 18 mM boric acid-0.3 mM EDTA pH 8.1, voltage 8 kV, 10 psi sample injection; (b.) sample concentration 14 or 7 mg/ml Tf-Fe^{3+}-aziridine-carboxylate, background electrolyte 100 mM MES pH

6.1, voltages 5, 8, 10 kV, 10 psi sample injection; (c.) the samples are a mixture of 5 mg/ml Tf-Fe^{3+}-bicarbonate or Tf-Fe^{3+}-oxalate and 5 mg/ml iron free Tf, background electrolyte is 18 mM TRIS- 18 mM boric acid-0.3 mM EDTA pH 8.1, voltage 9 kV, 5 psi sample injection, glass capillaries coated with non-crosslinked polyacrylamide (PA). The standard experimental conditions are: glass capillaries with 50 μm ID, 280 mm effective length, 10 psi injection, 20 °C, on –tube detection at 280 nm. Samples concentration varies between 5-10 mg Tf-complex/ml.

Plate number and peak capacity which are considered as a dimensionless measure of separation efficiency were calculated based on the obtained electropherograms. The plate number in the case of uncoated capillary is in the range of 10^5-$2 \cdot 10^5$/m, and in the case of coated capillaries this number can achieve $3 \cdot 10^7$/m [9, 13]. At the analyzed conditions the non-crosslinked polyacrylamide coated capillaries generated the highest theoretical plate numbers, N=715.38 (Fig. 8) and accordingly to this, also the highest peak capacity, $n_c \approx 13.37$ (Fig. 9).

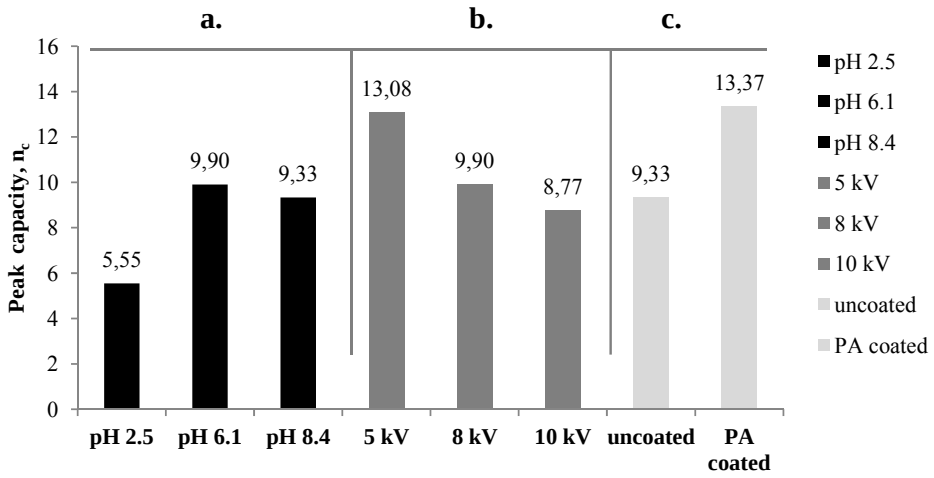

Fig. 9. Calculated peak capacity of the separation system changing the background electrolyte's pH (a.), voltage (b.) and coated and uncoated capillaries

The experimental conditions were the same as in the case of Fig. 8

The presented data shows the relations between the different separation conditions and its influence on the efficiency. Coated capillary, slower voltage provided better efficiencies especially using buffer with pH value in the range of pI of the protein. Theoretically, based on the calculated peak capacity and plate numbers the best separation condition can be achieved using background electrolyte with pH=6.1, 5 kV and coated capillary. Even though this predicted

conditions proved to be the optimal in the case of the uncoated capillary, it is not feasible in the case of coated capillary, due to the sensibility of the polymer coating (non-crosslinked-polyacrylamide) to the low pH.

3. Conclusions

Capillary zone electrophoresis is the most frequently used technique for protein samples separation. A simple CZE method was developed to separate the Tf-iron(III)-bicarbonate, Tf-iron(III)-oxalate, Tf-iron(III)-aziridine-carboxylate complexes. During the experiments we used coated and uncoated glass capillaries, background electrolytes with different pH, and voltages. Decreasing the voltage and using a background electrolyte with pH=6.1 generated the highest separation efficiency in the case of uncoated capillary. This approach resulted in greatly increased peck capacity ($n_c \approx 13.08$) and theoretical plate numbers (N=684.49). The highest separation of the transferrin samples was achieved using coated capillary, although the best condition achieved by uncoated capillary cannot be applied due to the polymers sensibility to low pH values.

Based on the obtained electropherograms and calculated data we suggest separation of transferrin samples by high performance capillary zone electrophoresis technique using non-crosslinked-polyacrylamide coated glass capillary, thus eliminating the electroendosmotic flow inside the capillaries.

Summarize the obtained electropherograms and calculated data we suggest to eliminate totally the electroendosmotic flow inside the capillaries using polymer treated or polymer coated glass capillaries for protein samples separation with high performance capillary zone electrophoresis technique.

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