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Short communication

Observation of interactions of human serum components with transferrin by affinity capillary electrophoresis

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Abstract

Interaction of human transferrin (TF) with human serum components was investigated by affinity capillary electrophoresis. It was found that any peaks of human serum protein fractions did not give migration time change on addition of intact TF to running buffer (50 mM phosphate buffer, pH 7.5), whereas two peaks belonging to α-globulin fraction showed marked acceleration upon addition of desialylated TF. These results provide strong evidence that the sialic acid residue in TF masks its binding ability to serum proteins. The association constants of desialylated TF to these interactive components, estimated based on the double reciprocal plot of migration time change *vs.* glycoprotein concentration, were at a high level of $10^7\ M^{-1}$. TF is well known as a ferric ion transfer protein, and hence formation of this protein might be changed by ferric ion. The presence of iron(II) played no essential role in this interaction, though its influence was not negligible.

Keywords: Affinity capillary electrophoresis; Human serum; Transferrin; Association constant; Modified capillary

1. Introduction

Affinity capillary electrophoresis (ACE) gives one of the separation modes of capillary electrophoresis, useful for separation of pharmaceutical and biological substances. It is a unique mode of separation performed in buffers containing macromolecules, mostly proteins. For example, Arai et al. [1] and Sun et al. [2,3] separated drug enantiomers using bovine serum albumin as a chiral selector. Valtcheva et al. [4] reported separation of β-blockers in the presence of cellulase. Vespalec et al. [5] described separation of amino acids and monobasic carboxylic acid enantiomers in a buffer containing human selum albumin.

The importance of ACE is, however, rather in its high capability in binding studies. Avila et al. [6] developed a competitive binding method for the interactions between carbohydrates and proteins. Studies by Kuhn et al. [7] on the interaction of lectins and carbohydrates are also noticed. Hong et al. [8] showed the utilities of ACE using oligosaccharide probes labeled fluorescently at the reducing end. We also have made a series of studies focusing on method development for association constant (K_a) estimation [9–11]. We established protocols for the estimation of K_a in normal (protein as sample/ligand as additive in running buffer) and reverse (vise versa) systems, and pointed out the advantages of individual systems. The binding studies by ACE are based on the principle that apparent mobility of either solute. Such change is observed for not only a single solute but multiple ones, and therefore is a powerful tool for simultaneous estimation of K_a values to a common ligand or protein under the same conditions. The simplicity of this procedure is that it does not require immobilization of either interactant to a solid phase as in ELISA, affinity chromatography and surface plasmon resonance, is another merit of ACE. Hyphenation to mass spectrometry is a further fascinating advantage of this method. Thus, ACE has recently been used for binding studies as the simplest method that allows reliable K_a estimation using only small amounts of both interactants. Here we should draw attention that migration time change is a sign of interaction, and we can readily recognize interaction occurring in a capillary and even separate the bound species at the outlet of the capillary.

Based on this much simpler principle we have undertaken another series of work for search and characterization of substances interactive to proteins or pharmaceutical and biological molecules. For example, we reported a search for serum protein-binding oligosaccharides, based on the delay of the disaccharide

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peaks in serum-containing buffer [12]. In this work it was found that disaccharide-binding serum protein peaks were also delayed in a disaccharide-containing buffer at the same time. The principle and procedure are quite simple and the disaccharides and proteins interactive to each other can easily be picked up, as if a fish is caught using bait in fishing. We have extended this kind of work to search for other substances interactive to macromolecules in tissues and body fluids, and observation of biologically important phenomena, because we can expect developing biologically active substances from biological samples. Human serum contains many kinds of glycoproteins playing important functions. Among these proteins transferrin is a suitable candidate for a target, because it is one of the most popular and simple glycoconjugate protein.

This paper describes another example of the application of this 'fishing' technique, in which the binding behavior of transferrin, an iron-transporting serum glycoprotein, was observed in relation to its desialylation.

2. Experimental

2.1. Materials

A pooled human serum obtained from BIO Whittaker (Walkersville, Maryland, USA) was used in this work. The specimens of transferrin and apo-transferrin (apo-TF) from human serum were purchased from Sigma (St. Louis, MO, USA). *N*-acetylneuraminidase from *Arthrobacter ureafaciens* obtained from Nakalai Tesque (Nakakyo-ku, Kyoto, Japan) was used as a sialydase sample. Hexadimethrine bromide (Polybrene) and polyacrylic acid (for capillary coating) were obtained from Aldrich (Milwaukee, CA, USA) and Wako (Osaka, Japan), respectively. All other chemicals were of the highest grade commercially available. Deionized and glass ware distilled water was used for preparing running buffers and sample solutions.

2.2. Coating of capillary

Polybrene/polyacrylic acid double layer coating was carried out according to a procedure of Katayama et al. [13] with some modification, briefly as follows. A piece of 50-cm portion of 50-μm i.d. fused silica capillary was cut out from a roll obtained from Polymicro Technologies (Poenix, AZ, USA). It was rinsed with 1 M sodium hydroxide followed by water for 5 min each, then with a 5% aqueous Polybrene solution for 15 min. A Polybrene layer was formed by this treatment. The capillary was subsequently washed by an aqueous 3% polyacrylic acid solution for 15 min to make the second layer. The double layer coated capillary was washed with water for 5 min.

2.3. ACE

ACE was performed using a Photal CAPI-3200 apparatus equipped with a vacuum hydrostatic sample introduction device, an air circulation thermostated capillary oven, and a diode array UV detector. A double layer coated capillary was installed on a capillary cassette and the cassette was fixed onto the apparatus. The capillary oven was kept at 25 ± 0.1°C. The capillary was rinsed with 0.5% polyacrylic acid solution for 2 min, followed by a running buffer for 8 min before each run. Running buffer solutions were prepared by dissolving TF or desialylated TF at various concentrations in a 50 mM phosphate buffer, pH 7.5. ACE was carried out by applying a constant voltage of 15 kV between both ends of capillary. Detection was carried out by monitoring UV absorption at 200 nm.

2.4. Sialidase digestion of TF

TF (200 μg) was dissolved in 80 μL of water, and 20 μL of a sialidase solution prepared by dissolving *N*-acetylneuraminidase in a 5 mM acetate buffer (pH 5.0) at a concentration of 100 μU/100 μL, was added. The mixture was incubated at 37 °C for 24 h, then lyophilized. The residue was dissolved in a 50 mM phosphate buffer (pH 7.5), and diluted to appropriate concentrations.

3. Results and discussion

3.1. Electropherograms of human serum in a TF-containing electrophoretic solution

A serum sample was prepared by diluting human serum to a 10-fold volume with distilled water, and the diluted serum was introduced to the capillary anodic end by hydrostatic introduction with a 2.5-cm gap of reservoir levels for 30 s. Capillary electrophoresis of proteinaceous samples in an untreated fused silica capillary caused varied migration times, peak responses, and peak shapes due to adsorption of the sample on the capillary inner wall. However, use of the double layer coated capillary solved this problem, giving improved reproducibility of migration time and symmetrical peak shapes. Fig. 1 shows electropherograms of human serum proteins in the absence and presence of TF.

In each electropherogram diluted human serum gave 5 peaks of globulins and a large peak of albumin together with a peak of a neutral marker (cinnamyl alcohol), were separated from each other. Addition of TF (Fig. 1b and c) did not cause migration time change, indicating no interactions between TF and these proteins.

3.2. Influence of desialylation on serum protein peaks

TF is well known as a glycoprotein having *N*-linked oligosaccharide chains with an *N*-acetyl neuraminic acid (NANA) residue at each non-reducing end. Scheme 1 represents the major glycoform of TF. The NANA residues are easily released by sialidase digestion under slightly acidic conditions.

In order to examine the influence of releasing NANAs TF was desialylated by sialidase digestion and TF as an additive to running buffer was replaced by desialylated TF. Fig. 2 shows the electropherograms of diluted human serum in the presence and absence of desialylated TF.

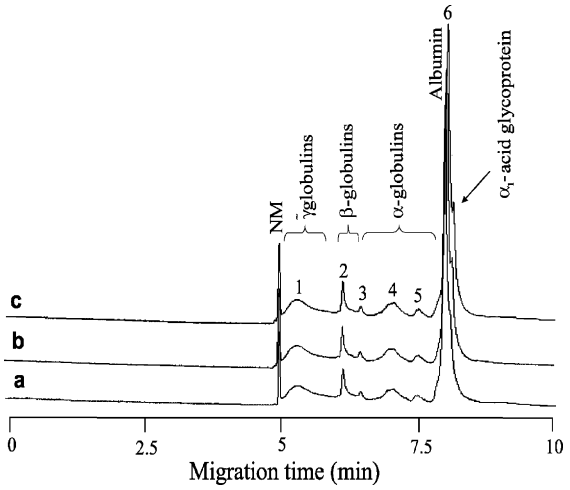

Fig. 1. Electropherograms of human serum by capillary electrophoresis using transferrin-containing electrophoretic solutions. Capillary, PB/PAA-coated fused silica (50 μm i.d., 50 cm); electrophoretic solution, 50 mM phosphate buffer, pH 7.5 containing transferrin at the concentrations of 0 (a), 10 (b) or 30 μg/ml (c), respectively; applied voltage, 15 kV; temperature of capillary oven, 25 °C; detection, UV absorption at 200 nm.

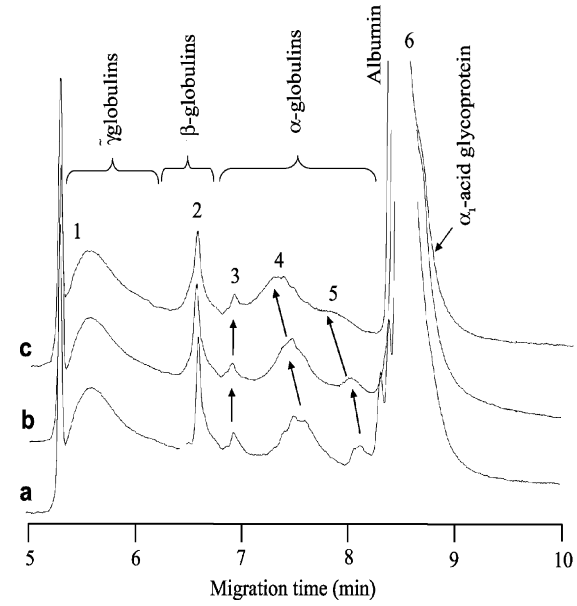

Fig. 2. Electropherograms of human serum by capillary electrophoresis using desialylated transferrin-containing electrophoretic solutions. Electrophoretic solution, 50 mM phosphate buffer, pH 7.5 containing desialylated transferrin at the concentrations of 0 (a), 10 (b) or 30 μg/ml (c), respectively; other conditions are as in Fig. 1.

It is clearly shown that migration times of peaks 4 and 5 which belongs to the α-globulin fraction decreased as desialylated-TF concentration increased (Figs. 2 and 3), where other peaks did not give migration time changes. It is obvious that the proteins giving peaks 4 and 5 have specific affinity to desialylated TF.

3.3. Simultaneous determination of association constants of the interactive components to desialylated TF

ACE allows simultaneous determination of association constant (K_a), based on the principle that the mobility of the formed adduct is the sum of the free and bound species multiplied by their molar fractions. The details of this method were described in our previous paper [9]. The conclusion is that the double reciprocal plot of migration time change against additive concentration forms a straight line. K_a can be expressed as $(Bt_1 - 1)/At_1$ where A and B are the slope and the Y-intercept of the straight line, respectively, and t_1 designates the migration time of a protein in the absence of a ligand.

Thus, the migration time was measured accurately at the peak top position using an integrator and corrected for slight variation of electroosmotic flow, as described in our another paper [12]. The double reciprocal plot of $(t - t_1)$ *vs.* [desialylated TF] actually gave a straight line, where t_1 and t are the migration times of a protein peak in the absence and presence of desialylated TF, at a concentration of [desialylated TF], respectively.

Peaks 4 and 5 gave straight lines, $Y = -3.66 \times 10^{-7}X - 4.09$, $R = 0.999$ and $Y = -3.74 \times 10^{-7}X - 6.97$, $R = 0.996$, respectively, where R means correlation coefficient. The obtained K_a values for peaks 4 and 5 were $1.08 \times 10^7\ M^{-1}$ and $1.83 \times 10^7\ M^{-1}$, respectively. In order to check the influence of the sialidase added, diluted human serum was analyzed in running buffer containing *N*-acetylneuraminidase at the same concentration as above. The result shows that the sialidase did not give any influence on the estimation of K_a. Since TF is important as an iron-transporting protein, the above-mentioned

Scheme 1. Structure of oligosaccharide observed from human transferrin.

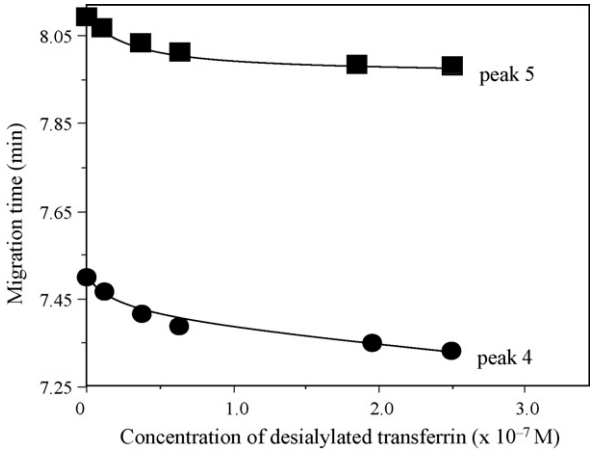

Fig. 3. Plots of migration times of peaks 4 and 5 *vs.* concentration of desialylated transferrin.

binding study was carried out using TF and desialylated TF-containing iron(II). However, we should know the significance of iron(II) in binding to serum proteins. Therefore, we examined the migration time change of serum proteins in the presence of desialylated apo-TF. Similar change of migration time was observed, and, the K_a values for peaks 4 and 5 were $1.38 \times 10^6\,M^{-1}$ and $7.98 \times 10^5\,M^{-1}$, respectively. These values are smaller than those for desialylated TF-containing iron ion (II) by 1–2 order of magnitude the difference is obviously due to the participation of the iron ion in binding to these proteins probably by coordination of the iron ion with the basic groups in the proteins. In conjunction with this finding we also examined the effect of addition of apo-TF on the analysis of serum proteins. However, we did not noticed any effect in this case.

4. Concluding remarks

We presented herein an example of search for substances interactive to serum components. We found that intact TF gave no influence on serum protein peaks, but desialylation caused marked change of migration time for two components belonging to α-globulin fraction. This reflects the importance of sialic acid residue in controlling affinity of TF to serum proteins. The K_a values of desialylated TF to serum proteins were as high as $10^7\,M^{-1}$, suggesting specific binding. Results of our previous work [12] shows that α-globulin components do not interact with a gentiobiose derivative, but interact with a lactose derivative. On the other hand, γ-globulin components interact with a gentiobiose derivative. Therefore, many kinds of globulins can recognize a structure of a saccharide binding to the non-reducing end of an oligosaccharide. In the work by Poulik [14] on diphtheria toxin, presence of a potent neuraminidase affects microheterogeneity of one of γ-globulin. This fact also supports our results. The presence of iron(II) did not give a crucial influence on the binding, though not to negligible extent. The identification of the interactive components needs further investigation. On-line MS studies are now in progress.

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