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Short communication

Separation of components of human globulins by capillary zone electrophoresis using a linear polyacrylamide-coated capillary

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Abstract

Eleven commercially available protein preparations from human serum were subjected to capillary zone electrophoresis (CZE) using a linear polyacrylamide-coated capillary at pH 7.4. Transferrin, complement C3 and C-reactive protein were each separated into one major peak and several minor peaks. α_1-Antitrypsin was separated into two major peaks and three minor peaks. α_2-HS-glycoprotein showed four major peaks with a leading shoulder. Haptoglobin, α_2-macroglobulin, α_1-acid-glycoprotein and prealbumin were detected as relatively wide peaks. Ceruloplasmin showed one major peak with notches, and two minor and several notched peaks. Only low density lipoprotein showed no peaks. A mixture of five of the protein preparations was separated into individual components, as well as individual isoforms. When the same mixture was analyzed by CZE using an uncoated capillary, a much poorer resolution was obtained. Application of this CZE system to albumin-depleted serum demonstrated that it is very useful for analyzing globulin components in serum. ©1997 Elsevier Science B.V.

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1. Introduction

Zone electrophoresis on cellulose acetate membranes (cellulose acetate electrophoresis; CAE) and agarose gels (agarose gel electrophoresis; AGE), which are currently used in most clinical laboratories, separate serum protein components into five or more zones. The major zones are albumin, alpha-1 (α_1), alpha-2 (α_2), beta (β) and gamma (γ). Clinical diagnostic information can be obtained from variations in the relative contents of these zones and from electrophoretic patterns, such as peak shapes and peak symmetries (obtained by scanning stained zones).

Recently, capillary zone electrophoresis (CZE) using an uncoated capillary has been used by many researchers for separation of human serum proteins [1–10]. The results showed that CZE gives the same major fractional zones as those observed in CAE and AGE [3–6,8,9], and also provides comparable or better resolution. The use of a higher strength proprietary buffer [1] and a buffer prepared by considering the pK_a values of the components of the buffer [7] were found to separate serum proteins into more than ten zones or inflections, each of which corresponded to various serum protein components.

In contrast to the current use of an uncoated capillary for CZE of serum proteins, a capillary with a neutral polymer coating has been used in a small number of studies. These include an early study by Jorgenson and Lukacs [11] and more recent studies by Hjertén [12], Hjertén and Johansson [13] and Huang et al. [14]. In the study by Jorgenson and Lukacs, it seems that the capillary coating used was

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insufficient, because electroosmotic flow can be seen in the capillary in Fig. 9 of that study. Because of the problem of protein adsorption on uncoated capillary wall, an uncoated capillary requires high [15,16] or low [17] pH buffers to obtain protein separation. However, a capillary coated with a neutral polymer makes it possible to separate proteins around physiological pH with high efficiency [18]. In addition, separation of serum proteins at physiological pH would appear to be more meaningful than that at non-physiological pH values [11–14]. In this study, human serum globulin components were subjected to CZE with a capillary with a neutral polymer coating at such a pH value.

2. Experimental

2.1. Materials

The following protein preparations from human serum or plasma, all commercially obtained, were used: albumin, holo-transferrin, prealbumin (PA), α_1-acid-glycoprotein, ceruloplasmin (CP) and complement C3 (C3) (Sigma, St. Louis, MO, USA); haptoglobin and α_2-HS-glycoprotein (HSGP) (Athens Research and Technology, Athens, GA, USA); α_2-macroglobulin (Organon Teknika Corporation, Durham, NC, USA); α_1-antitrypsin (Chemicon International, Temecula, CA, USA); C-reactive protein (CRP) (Incstar, Stillwater, MI, USA); low density lipoprotein (BioPur, Bubendorf, Switzerland). Serum samples were obtained from the central clinical laboratory of Kinki University Hospital. Ultrafree-C3LGC filtration tubes (molecular mass cut-off, 10 000) were obtained from Millipore (Tokyo, Japan). HiTrap Blue (1 ml) was obtained from Pharmacia Biotech (Tokyo, Japan). Fused-silica capillary tubing (I.D. 75 μm; O.D. 375 μm) was obtained from Otsuka Electronics (Hirakata, Osaka, Japan). γ-Methacryloxypropyltrimethoxysilane (LS-3380) was obtained from Shin-Etsu Silicon Chemicals (Tokyo, Japan). Electrophoresis grade acrylamide was from Daiichi Pure Chemicals (Tokyo, Japan). HEPES, and Tris were obtained from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals used were of guaranteed grade, and purchased from Wako.

2.2. Methods

2.2.1. Sample preparation

Aliquots of protein preparations (2–5 mg/ml) supplied in high salt concentration were desalted by ultrafiltration (Ultrafree-C3LGC) with addition of a suitable volume of 10 m*M* HEPES–Tris buffer, pH 7.4. This desalting was repeated twice. Finally, protein solutions of about 1 mg/ml in 10 m*M* HEPES–Tris were obtained. For salt-free lyophilized protein preparations, the proteins were dissolved in 10 m*M* HEPES–Tris buffer at a concentration of 1 mg/ml. Five preparations, α_1-acid-glycoprotein (AGP), α_1-antitrypsin (AT), haptoglobin (HP), α_2-macroglobulin (α_2M) and transferrin (TF), were mixed, and then similarly desalted with the 10 m*M* buffer. The four proteins except AGP were adjusted to a concentration of 1 mg/ml, and AGP was adjusted to 0.4 mg/ml. These samples were applied to CZE using both coated and uncoated capillaries, and to non-reduced SDS–PAGE.

2.2.2. Capillary zone electrophoresis

CZE was performed on a Quanta 4000 capillary electrophoresis system (Nihon Waters, Tokyo). For CZE with a coated capillary, the protein samples were introduced from the cathode end of the capillary by gravity, whereas for CZE with an uncoated capillary, the electrodes were reversed, and the samples were introduced from the anode. The electrophoresis was carried out with a running buffer of 0.1 *M* HEPES–Tris, pH 7.4, (prepared by mixing 0.1 *M* HEPES and 0.1 *M* Tris) at room temperature (24–26°C) at a constant voltage of 10 kV on a capillary with a total length of 33 cm (effective length of 25 cm). Separations were monitored at 214 nm. The capillary was coated according to the method of Hjertén [19] with some modifications [20]. The coating was done with linear polyacrylamide formed by mixing 500 μl of 7% (w/v) acrylamide, 5 μl of 10% (w/v) ammonium persulfate and 5 μl of 10% (v/v) N,N,N′,N′,-tetramethylethylenediamine (TEMED) in 50 m*M* sodium phosphate, pH 7.0.

2.2.3. Examination of preparations by non-reduced SDS–PAGE

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS–PAGE) [21] was

used to check the purity of the preparations. SDS–PAGE was performed under non-reducing conditions using gels of 6% T-2.7% C and 10% T-2.7% C at a constant 100 V and at room temperature. The staining solution contained 0.3% (w/v) Coomassie Brilliant Blue R-250, 10% (v/v) acetic acid and 25% (v/v) 2-propanol. The gels were destained with 10% (v/v) acetic acid containing 10% (v/v) methanol at 50°C.

3. Results and discussion

3.1. Electrophoresis of individual components

Fig. 1 shows the (CZE) electropherograms of ten protein samples performed in 0.1 *M* HEPES–Tris at pH 7.4. Each sample had an apparently different electrophoretic mobility and an unique peak profile. The migration order was as follows: prealbumin (PA), α_1-acid-glycoprotein (AGP), ceruloplasmin (CP), α_1-antitrypsin (AT), α_2-HS-glycoprotein (HSGP), haptoglobin (HP), α_2-macroglobulin (α_2M), transferrin (TF), complement C3 (C3) and C-reactive protein (CRP). The order was, on the whole, consistent with that observed with electrophoresis using supported materials (cellulose acetate membrane and agarose gel), as well as an uncoated capillary, except that the migration order of CP (c) and AT (f) was reversed. AT and CP are commonly identified in the α_1 and α_2 zones, respectively, on CAE and AGE. TF (g) and C3 (j), both of which are β-globulins, had clearly different mobilities, resulting in good resolution of these peaks. The same resolution can be seen also in AGE [22]. AT (f) was separated into several isoforms (variants). Separation of variants was also found in TF (g) and HSGP (h). The high resolution observed in the three globulin components (AT, TF and HSGP) revealed that CZE with a coated capillary was as effective in protein separation as isoelectric focusing, assuming proteins are separated on the basis of their charge density.

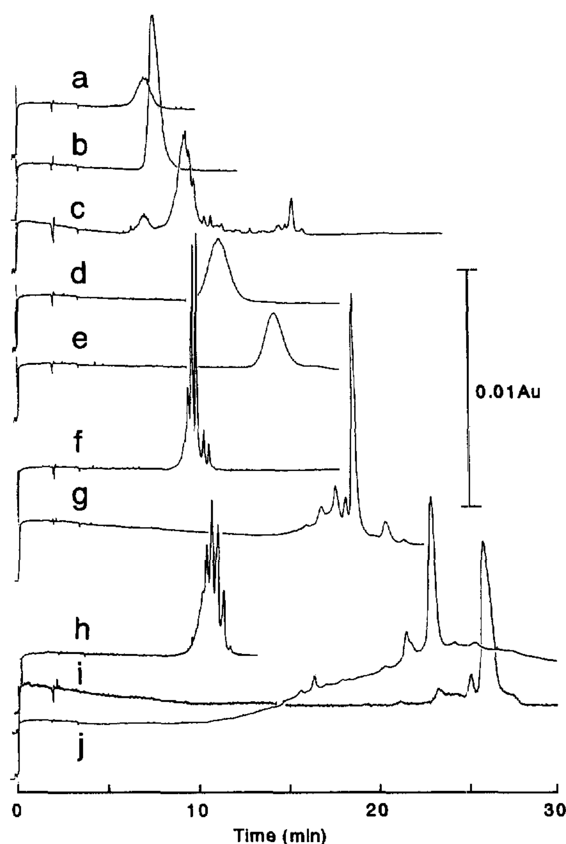

Fig. 1. Electrophoresis of various protein components from human serum. (a) prealbumin, (b) α-1-acid-glycoprotein, (c) ceruloplasmin, (d) haptoglobin, (e) α-2-macroglobulin, (f) α-1-antitrypsin, (g) transferrin, (h) α-2-HS-glycoprotein, (i) C-reactive protein, (j) complement C3. Sample introduction and concentration: 6 s except C-reactive protein (2 s) by gravity and 1.0 mg/ml, respectively. Electrophoresis: coated capillary with 0.1 *M* HEPES–Tris, pH 7.4, at −10 kV. Detection, 214 nm.

3.2. Inspection of protein contaminants

Fig. 2 shows the electrophoretic patterns for five of the preparations used in Fig. 1. Under non-reduced conditions in the presence of SDS, proteins are expected to migrate mainly by a size exclusion effect. AT, TF and AG, which are well known to have electrophoretic variants because of differences in amino acids and carbohydrate compositions, migrated in single bands (Fig. 2B). α_2M (Fig. 2A, e) separated into two bands, probably corresponding to the dimer (360 kDa) and tetramer (720 kDa) forms. HP (Fig. 2A, d) showed several bands, indicating the presence of HP phenotype variants which separated due to structural variations. Based on the non-reduced SDS–PAGE results, the five preparations appeared to be free of contaminants.

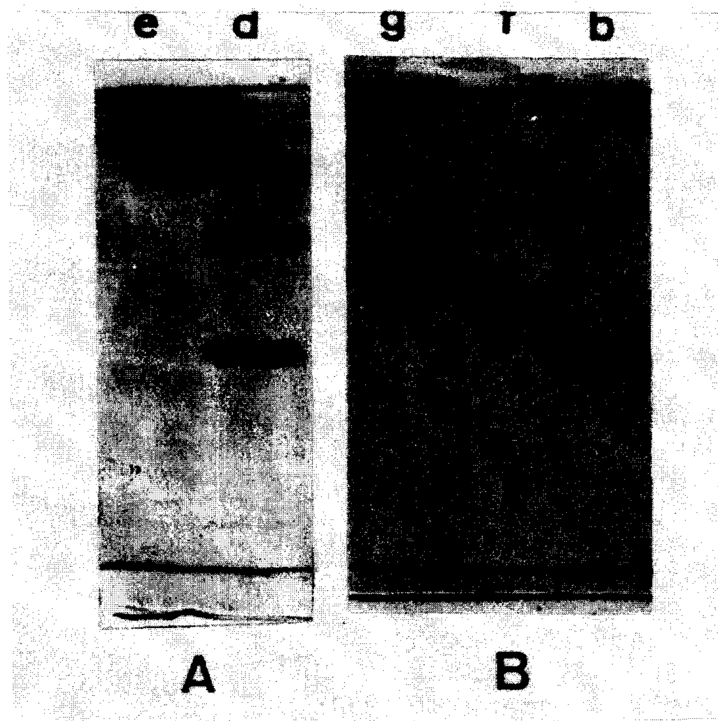

Fig. 2. Non-reduced SDS–PAGE of five preparations used as model proteins. Polyacrylamide: A, 6% T-2.7% C; B, 10% T-2.7% C. The lanes are labelled with the same letters as in Fig. 1. Amount applied (μg), from left to right, 2.5 (e), 5 (d), 1.5 (g), 1.5 (f) and 1.5 (b). Electrophoresis was done at 100 V and at room temperature.

3.3. Separation of mixed components

The five proteins, AGP, AT, HP, α_2M and TF, which did not overlap with each other on the electropherograms (Fig. 1), were mixed and then subjected to CZE using a coated capillary (Fig. 3A). The mixtures were clearly separated into individual components within 20 min. AGP (b) and AT (f), both of which belong to the α_1-globulin family and both of which are located in the α_1 zone on CAE and AGE, were resolved. The two α_2-globulins, HP (d) and α_2M (e), were also resolved. CAE and AGE do not resolve these globulins within a zone. Moreover, the respective peak profiles were the same when they were examined independently (Fig. 1, b, d, e, f and g). The complete separation of the five proteins and the clear separation of variants shown in Fig. 3A could not be obtained by CZE using an uncoated capillary at pH 7.4 (Fig. 3B) or at pH 10.0 (50 m*M* sodium tetraborate) (data not shown). Separations similar to those shown in Fig. 3B using an uncoated capillary in solutions of high pH have been obtained by Chen [1] and Dolník [7], for whole serum. They were unable to separate the variants. These results show that CZE with a coated capillary at a neutral pH range can give better resolution than that obtained with an uncoated capillary.

Comparison of the two electropherograms in Fig. 3 demonstrates that CZE with a coated capillary is useful not only for the separation of mixtures of globulins, but also for the separation of protein phenotype variants. The high resolution obtained at physiological pH seems to be due to slight differences in protein charge, which would result from differences in the degree of proton dissociation of the side chains of different amino acid residues (pK_a values range from 5 to 10). Such variations in charge density would not be expected for proteins in solutions of low and high pH. It is thus apparent that CZE carried out at pH 7.4 with a coated capillary

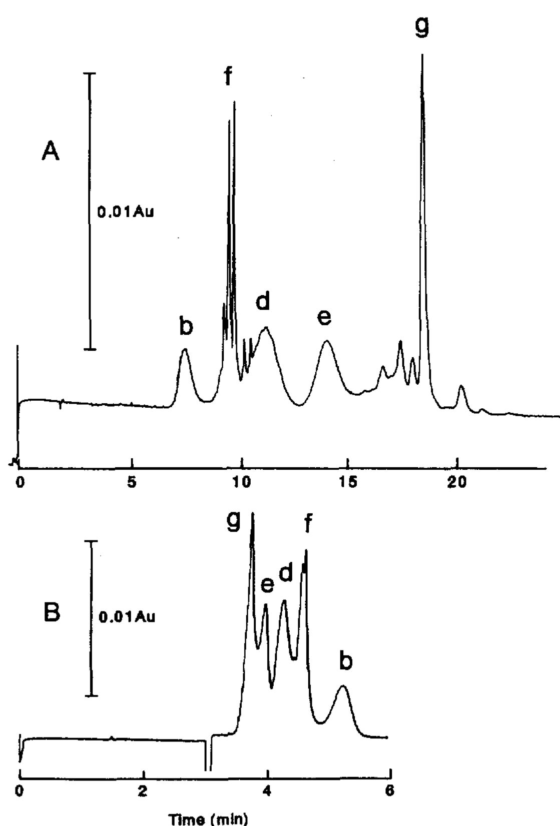

Fig. 3. Effect of capillary coating on the separation of globulin components using CZE. (A) Coated capillary; (B) uncoated capillary. Peak labels are defined in the legend of Fig. 1. Protein concentration, 1 mg/ml except peak b (α-1-acid-glycoprotein, 0.4 mg/ml). Introduction, 6 s by gravity. Except for the positive voltage (+10 kV) used in B, the operating conditions were the same as those used in Fig. 1.

can separate globulins with high resolution. However, albumin was found to migrate to the HP zone (data not shown). Thus, in order to detect serum globulins with a resolution as good as that shown in Fig. 3A, it is necessary to first remove serum albumin, which generally represents more than half of the serum proteins. Therefore, preparations of albumin-free serum, which could be obtained by group separation of serum proteins using a HiTrap Blue column, were analyzed by CZE. Fig. 4 shows the preliminary results for four preparations obtained from the sera of two normal subjects (A and B) and two patients with hyperlipemia, diabetes mellitus and craniopharyngioma (C) and with liver cirrhosis (D). The peak profiles of the normal preparations were different from those of the patient preparations, and there was also a difference between the two patient preparations. Peak i, which is probably is CRP, was observed only in the two patient preparations. The CZE system thus appears to offer important information on the variation in quality and quantity of the individual components of serum globulins.

In order to clearly interpret a CZE electropherogram, in addition to the removal of impurities, it is also important to understand whether the protein of interest is in its native form or denatured, and especially whether it has undergone peptide bond cleavage with a resulting loss of charged amino acids or deamidation of amino acid side chains. Such alterations may not be detected by non-reduced SDS–PAGE (Fig. 2). It would seem likely that the various processes used to purify a protein would cause some damage [20]. If this is the case, preparations having the same names as those used in this study, but which differ in either the source or lot, may not show the same electrophoretic behavior as that reported here. If another preparation is different in amino acid composition or in the degree of modification of amino acid side chains, it will probably have a different electropherogram. Such differences are seen among bovine serum albumin preparations on CZE with a coated capillary performed at neutral pH [20].

Finally, these results show that the use of CZE with a coated capillary makes it possible to separate the various components of globulins, as well as or in some cases more completely than, can be achieved by CAE or CZE with an uncoated capillary (both of which commonly separate globulins into only four groups) or AGE (which commonly separates globulins into only five groups) [22]. However, a disadvantage is that this system requires a much longer time than is required by the uncoated CZE systems of Chen [1] and Clark et al. [9], which can be as short as 120–200 s. Further studies using the sera of normal and diseased subjects are needed to show the usefulness of the CZE system, and are now in progress.

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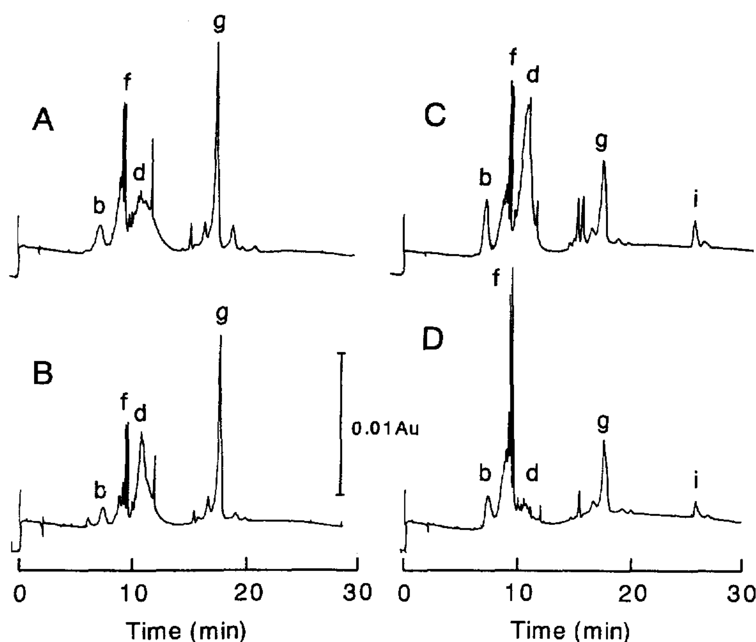

Fig. 4. CZE of albumin-depleted serum preparations from normal and diseased human subjects. During the preparation process, samples were diluted two times compared to original sera. Introduction, 5 s by gravity. Peak labels are defined in the legend of Fig. 1. (A) Normal (47-yr-old male); (B) normal (13-yr-old female); (C) patient with hyperlipemia, diabetes mellitus and craniopharyngioma (56-yr-old male); (D) patient with liver cirrhosis (56-yr-old male). The operating conditions were the same as those used in Fig. 1.

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