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Two−dimensional separation of human plasma proteins using agarose gel isoelectric focusing followed by SDS capillary electrophoresis

Jun Mukai, Nao Yamaguchi and Takashi Manabe

Department of Chemistry, Faculty of Science, Ehime University, Matsuyama, JAPAN

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Abstract

Two−dimensional separation of human plasma proteins combining agarose gel isoelectric focusing and SDS capillary electrophoresis was examined. After IEF, the IEF gel was dissected to 32 slices, then the proteins were extracted from the gel slices and were subjected to SDS−CE. Each electropherogram was converted into a density profile, the density profiles were arrayed in the order of IEF slice number and a 2−D density pattern was constructed. On the 2−D density pattern, about 90 protein "spots" were detected in pI range from 3.5 to 8.0 and molecular mass range from 20 kDa to 500 kDa. When the density pattern was compared with a pattern of 2−D gel electrophoresis, major protein spots could be correlated. This technique has the advantages in post−electrophoresis data processing when compared with conventional 2−D gel electrophoresis; 1) protein quantity can be obtained without staining, 2) molecular masses can be estimated from the migration time, 3) the separation results can be stored as digitized data.

Keywords: two−dimensional separation, agarose gel isoelectric focusing, plasma proteins, SDS capillary electrophoresis.

1 Introduction

Two−dimensional polyacryiamide gel electrophoresis (2−D PAGE) has been widely used for protein separation because of its high resolution; more than one thousand proteins or polypeptides can be separated according to the differences of their isoelectric point (pI) and molecular mass. However, 2−D PAGE involves many steps of manual handling, sample injection, setting gel molds in an electrophoresis apparatus, removal gels from the molds, staining and destaining, etc. For quantitative analysis of protein on stained gels, more steps of manual handling are required in computer−assisted image analysis, densitometry [1−3]. On the other hand, there are non−gel separation methods of proteins such as HPLC and capillary electrophoresis (CE). Although these methods have an advantage that they can be automated, it is difficult to separate complex protein mixtures into individual protein peaks or fractions employing only one of these separation methods [3]. Therefore, multi dimensional separation systems have been reported for protein analysis, employing a non−gel separation method at the last dimension of the separation [4−8]. However, it is preferable to combine a method which separate proteins according to pI differences with the one which separate according to molecular mass in order to correlate the analysis results with the protein information accumulated during these 25 years using 2−D PAGE.

We have established the conditions of SDS−CE, using linear polyacrylamide (LPA) as a sieving matrix, to separate human plasma proteins according to their size differences [9]. About 30 UV (230 nm) absorbing peaks and shoulders were detected within 60 min when fused silica capillaries of 30 cm effective length and an automated CE apparatus were employed. These results showed that this method is suited as the last step of multi−dimensional separation of proteins. In this paper, we report on two−dimensional separation of human plasma proteins combining agarose gel isoelectric focusing (IEF) with SDS−CE. Proteins were separated under non−denaturing conditions in an agarose gel IEF column, the gel was dissected into 32 slices, then the proteins were extracted from the gel slices and subjected to SDS−CE. The multiple CE pat-

Corresponding Author: Dr. Takashi Manabe, Department of Chemistry, Faculty of Science, Ehime University, Matsuyama−City, 790−8577 JAPAN
Tel: 81(Japan)89 927 9609, Fax: 81(Japan)89 927 9590
E−mail: manabet@dpc.ehime−u.ac.jp

terns were transformed into density profiles and combined to synthesize a 2−D density distribution pattern, which enabled the comparisons of protein resolution of the 2−D separation system with that of a 2−D gel electrophoresis technique.

2 Experimental

2.1 Materials

Fused−silica capillaries (75 μm ID, 375 μm OD) were obtained from Polymicro Technologies (Phoenix, AZ, USA). Acrylamide, special grade for electrophoresis, was from Daiichi Pure Chemicals (Tokyo, Japan). *N, N'*−methylenebisacrylamide (Bis), Ammonium persulfate (APS) and *N, N, N', N'*−tetramethylethylendiamin (TEMED), all special grade for electrophoresis, Tris (hydroxymethyl) aminomethan (Tris) and sodium dodecyl sulfate (SDS), special grade for biochemistry, and glycine, ultra pure grade, were from Wako Pure Chemical Industries (Osaka, Japan). Coomassie Brilliant Blue R−250 (CBB) was from Fluka Chemie AG (Buchs, Switzerland). *N*−[Tris (hydroxymethyl) methyl] glycine (tricine) was from Dojindo Laboratory (Kumamoto, Japan). (3−Methacryloxypropyl)−trimethoxysilane was from Shin−etsu Chemical Industries. Carrier ampholytes Ampholine® (pH 3.5~10 and pH 3.5~5), agarose for IEF, were from Pharmacia Biotech (Uppsala, Sweden). Purified protein, human albumin was obtained from Sigma Chemical Company (St. Louis, MO, USA). Human plasma was obtained from healthy individuals.

2.2 Apparatus

A P/ACE system 2100 CE instrument (Beckman Instruments, Fullerton, CA, USA) was used for CE experiments and a laboratory−constructed 2−DE apparatus was used for 2−D gel electrophoresis combining agarose gel IEF and polyacrylamide gel SDS electrophoresis.

2.3 Agarose gel IEF and protein extraction

A human plasma sample (50 μL) was subjected to isoelectric focusing in the absence of denaturants employing 1% agarose column gels (0.5 Φ X 16 cm) which contained Ampholine® (pH 3.5−10 and pH 3.5−5 in final concentration of 2% and 0.5%) and 12% sorbitol. Isoelectric focusing was run at 2 mA/column constant current until a voltage of 500 V was reached and continued at 500 V constant voltage for 20 hours at 4℃. For the extraction of proteins from the agarose gel. One of the duplicated IEF gels was cut into 5 mm slices, the slices were numbered from 1 to 32, then each gel slice was finely crushed in a 100 μL aliquot of 1% SDS solution with an acrylic rod, and left for 1 h at room temperature. The gel suspension was centrifuged and the supernatant was subjected to CE (Figure 1). The remaining IEF gel column was used for the measurement of the pH gradient as described previously [10].

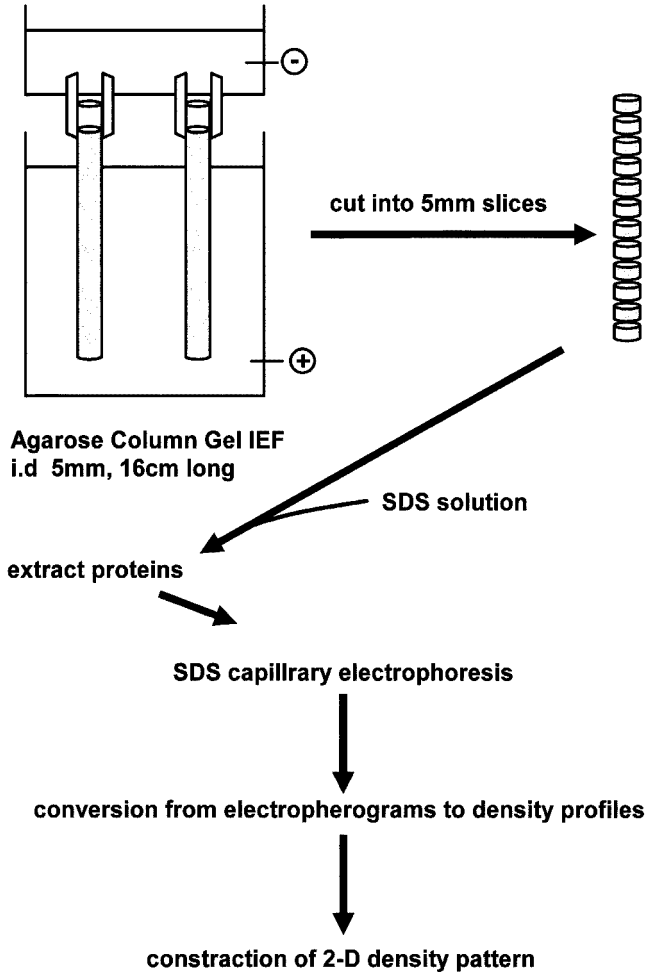

Figure 1. Scheme of the experimental procedures to construct a 2−D density pattern of human plasma proteins.

2.4 SDS capillary electrophoresis

2.4.1 Treatment of capillary inner surface

Fused−silica capillary tubing was cut into 41 cm segments with a detection window located 9 cm from one end. The capillaries were cleaned by successively filling with 0.1 M NaOH, deionized water, and methanol. The inner surface of the capillaries was then derivatized with (3−Methacryloxypropyl)−trimethoxysilane in a 1:1 methanol solution and left at room temperature overnight. The derivatizing solution was removed by passing methanol through the capillaries. A layer of noncross−linked polyacrylamide was then bonded to the inner surface of capillaries by filling them with degassed solution of 3% acrylamide/0.06% ammonium persulfate/0.06% TEMED and leaving them overnight at 30℃. The 3% polyacrylamide in the capillary was extruded by water pressure with a gas−tight microsyringe.

2.4.2 Preparation of linear poyacrylamide solution

The solution of linear polyaclylamide (LPA) to be filled in the

capillaries were prepared as follows: Acrylamide 0.8 g was dissolve in 5 mL water, the solution was mixed with 10.0 mL of a buffer solution (two−fold concentrated solution of electrode buffer solution), and the volume was adjusted to 20.0 mL. The solution degassed with an aspirator, poured in a 30 mL test tube, and left overnight at 30℃ for polymerization. The top layer of solution (4.0 mL) was removed and the remaining polyacrylamide solution was used to fill the capillary.

2.4.3 SDS capillary electrophoresis

The fused−silica capillary set to the apparatus was filled with a solution of LPA. Pre−electrophoresis was run for 20 min at the same electric field strength as that for separation. Sample was injected by electrokinetic force for 30 s and the separation was run at 5.55 kV (150 V/cm) electric field. In the case of human plasma sample, plasma solution was diluted 7−fold and mixed with an equal volume of 2% SDS solution.

2.4.4 Data processing and 2−D display of SDS−CE results

The profiles of SDS−CE obtained from thirty−two gel slices were converted to density profiles from the digitized UV absorbance data using a laboratory−made computer program. After subtracting the baseline values from the absorbance values, the absorbance data were correlated to 256 density steps. Then, the CE profiles were converted into density strips (*cf.* Figure 4), then the strips were arrayed in the order of IEF slice number. The position of albumin was used to further aligned the density profiles.

2.5 2−D gel electrophoresis employing agarose IEF gel in the first dimension and polyacrylamide slab gel in the second dimension

Agarose column gel (1% Agarose IEF®, 0.34 Φ X 16.5 cm) which contain 2% Ampholine® pH 3.5−10, 0.5% Ampholine® pH 3.5−5, and 13% sorbitol were used. A plasma sample was applied on the top of an IEF column gel and IEF was run at 0.6 mA/column constant current until a voltage of 500 V was reached and then at 500 V constant voltage for 20 h. The focusing IEF gel was transferred onto second−dimensional polyacrylamide slab gel (4.2−17.85% T gradient, 5% C 160 X 140 X 2.8 mm), equilibrated for 10 min with 2% SDS−0.01 M Tris−0.076 M glycine buffer (pH 8.3), and electrophoresis was run at 40 mA/slab constant current. Electrophoresis was continued until the band of BPB moved to about 5 mm from the bottom end of the slab gel. The gel was washed 50% v/v methanol 7% acetic acid for one hour before staining to remove carrier ampholytes, stained in 0.1% CBB R−250 in 50% v/v methanol/ 7% v/v acetic acid for over night, and destained in 20% v/v methanol/ 7% acetic acid for 6 h.

3 Results and discussion

3.1 Agarose gel IEF and protein extraction

We have selected agarose gel IEF among various techniques employing charge differences for protein separation, since it has the following advantages: 1) Isoelectric focusing is superior in resolution and in the separable pI range of proteins than ion−exchanging chromatography or other charge−based chromatographic or electrophoretic techniques. 2) Agarose gel has larger average pore size than polyacrylamide gel, then it is suited to separate large −molecular−mass protein complexes. The proteins can focus within shorter time, and the proteins can be easily extracted from the gel for the second separation step. Proteins were easily extracted from the gel by the procedure described in section 2.3. When the crushed gel slice was kept in a 1% SDS solution, the protein concentration in the supernatant became constant within 10 min. Therefore, we assumed that the recovery of proteins in this procedure to be calculated about 50%.

3.2 SDS−CE

Figure 2 A shows a SDS−CE pattern of human plasma proteins. The protein were separated into about 30 UV absorbing peaks and shoulders which appeared in molecular mass range from about 20 to 500 kDa. Major plasma proteins have been identified [9]. The extract solutions from the IEF gel slices were subjected to SDS−CE and one of the CE patterns was shown in Figure 2 B. The molecular mass value of proteins that were detected as UV absorbance peaks in electropherograms were calculated from their migration times using a standard curve, which had been prepared by plotting the migration times of purified proteins against logarithm of their molecular mass [9]. The UV−peak distribution in the pattern of Figure 2 B was quite different from that in Figure 2 A reflecting the separation of proteins in the step of agarose IEF. Figure 3 shows an overlapped presentation of CE patterns obtained from extract No.15 to 21 to show the changes in the separation patterns between different extracts. As indicated in Figure 3, most of the protein peaks in one CE pattern could be correlated with those in one of the adjacent CE patterns. Therefore, we assumed that the peaks appeared at the same migration time in two or more consecutive IEF gel slices might come from one protein species (*e.g.* marked by the arrows in Figure 3). Counting the number of protein species in the 32 CE patterns, about 90 species were detected. However, the presentation of the 32 CE separation patterns in the way shown in Figure 3 was too much complicated to get a perspective view of the plasma protein separation. Then, we tried to summarize the results by constructing a two−dimensional density pattern from the CE patterns.

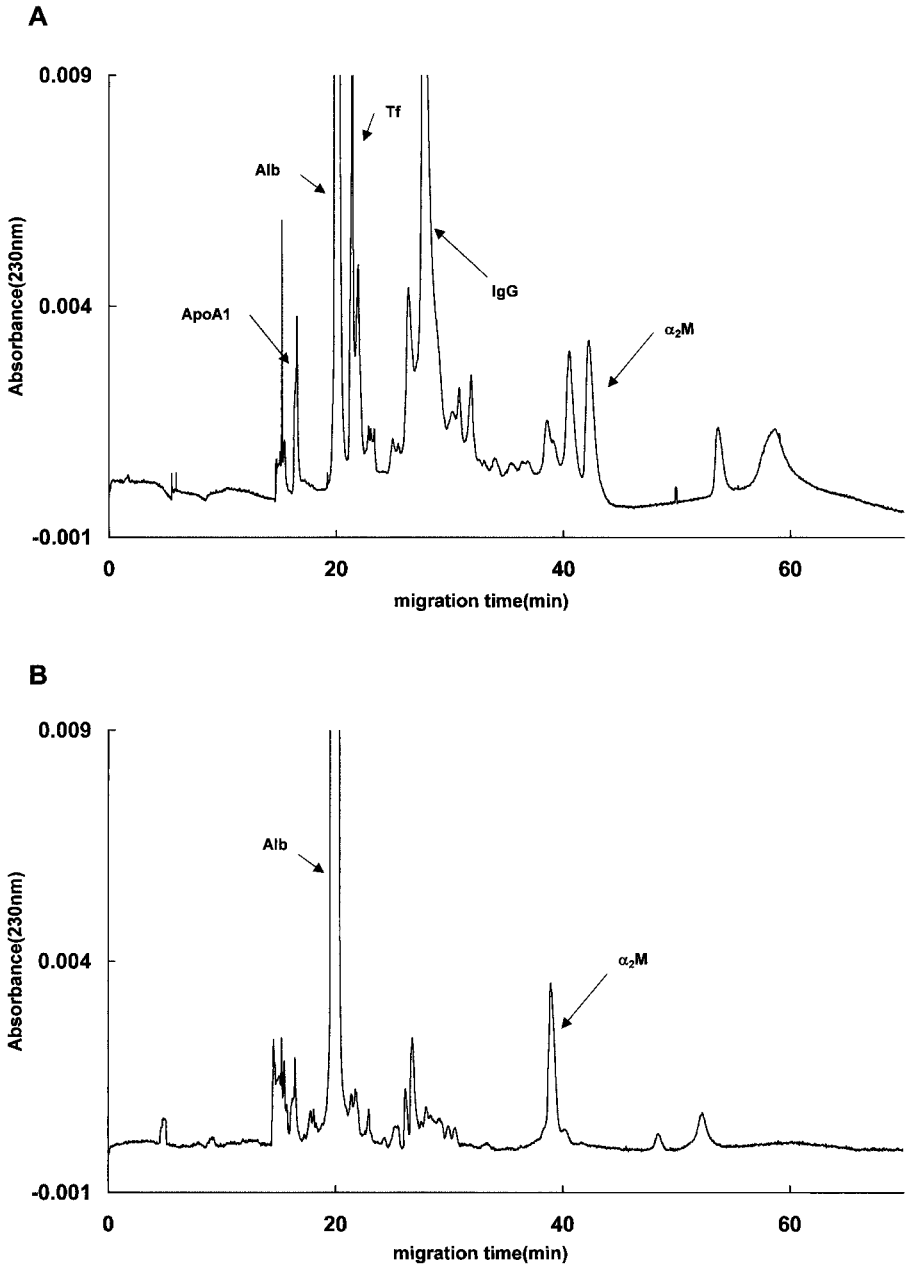

Figure 2. Examples of SDS capillary electrophoresis patterns; human plasma proteins (A), extracts from IEF gel slice No.15 (B). Capillary ID 75 μm; capillary effective length 30 cm; total length 37 cm; a buffering solution of 4% LPA was injected in the capillary and preelectrophoresis was done at 150 V/cm for 20 min; a sample solution was injected into capillary by electric field at 150 V/cm for 30 s. Abbreviations: ApoA 1, apolipoprotein A 1; Alb, albumin; Tf, transferrin; IgG, immunoglobulin G; α_2M, α_2−macroglobulin.

3.3 Conversion of CE patterns to density profiles and construction of a 2−D density pattern

Since the SDS−CE patterns of each extract were stored as digitized data, they were converted to the density profiles by a laboratory−made computer program. An example of the conversion from the SDS−CE pattern of slice number 21, is shown in Figure 4. The UV absorbance peaks in the CE profile (Figure 4 A) were converted into bands in the density strip (Figure 4 B). The density strips numbered from 1 to 32 were arrayed to construct a 2−D density pattern (Figure 5). As shown in Figure 5, the protein bands which showed the same migration time in consecutive density strips appeared as a protein spot. The quantities of protein recognized as the spots on a 2−D density pattern could be calculated by summing the peak area in the each electropherogram without pro-

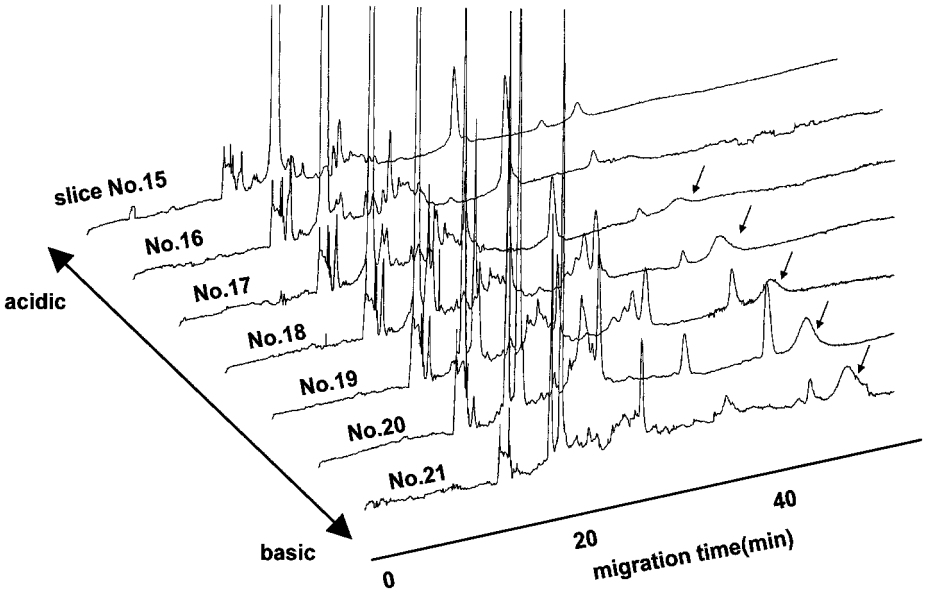

Figure 3. An overlapped presentation of SDS−CE patterns obtained from IEF gel slices No.15 to 21.

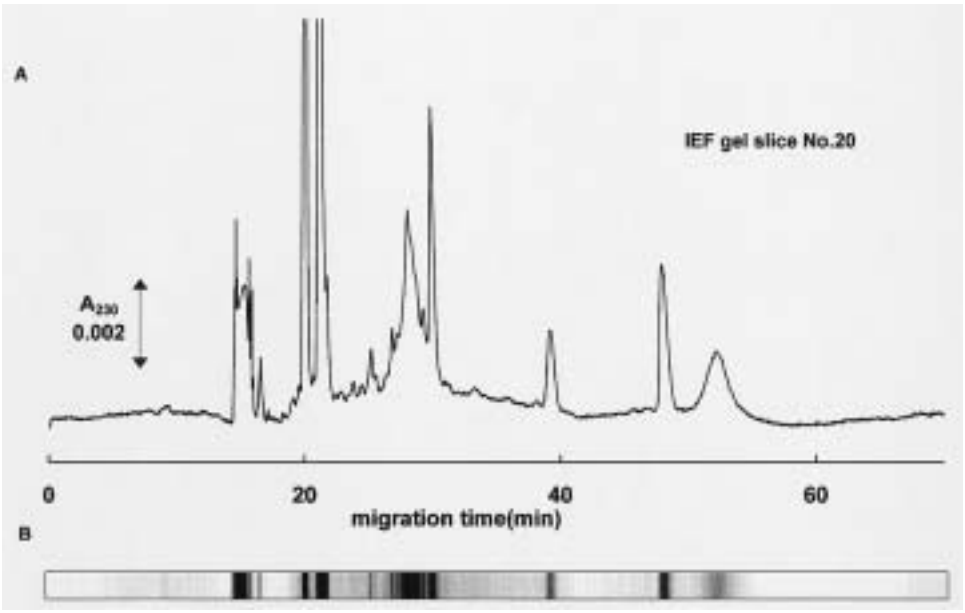

Figure 4. Conversion a SDS−CE pattern to a density profile. A SDS−CE pattern (A) was converted to a density strip (B) by laboratory−made computer program.

cedures such as gel staining and image analysis, which are requisites for 2−D PAGE gels.

3.4 Comparison of the 2−D separation method with a 2−D gel electrophoresis

3.4.1 Comparison of 2−D density pattern with a 2−D gel electrophoresis pattern

In order to evaluate the performance of the two−dimensional separation method, we tried to compare the 2−D density pattern with a 2−D gel electrophoresis pattern in which an agarose IEF gel was employed in the first dimension separation (section 2.5). The density pattern shown in Figure 5 was rotated for 90 °anti−clockwise and the values in pI axis were obtained by running duplicated agarose IEF gels and measuring the pH in the gel slices as described in section 2.3 and the values in Mr axis were obtained using a standard curve as described in section 3.2 (Figure 6 A). Major plasma proteins could be identified comparing the pI and molecular mass values to the data accumulated in our laboratory [2−4, 9]. Figure 6 B shows a 2−D gel electrophoresis pattern employing agarose gel IEF in the first dimension and SDS polyacrylamide gel

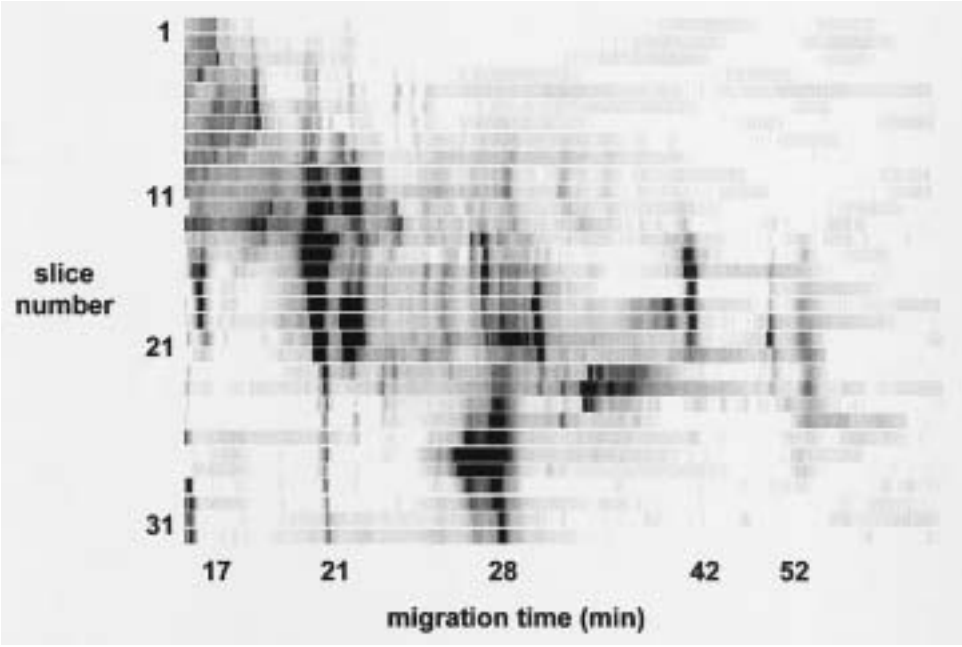

Figure 5. Construction a 2−D density pattern from the density strips. The density strips were arrayed in the order of slice number to construct the 2−D density pattern of human plasma proteins.

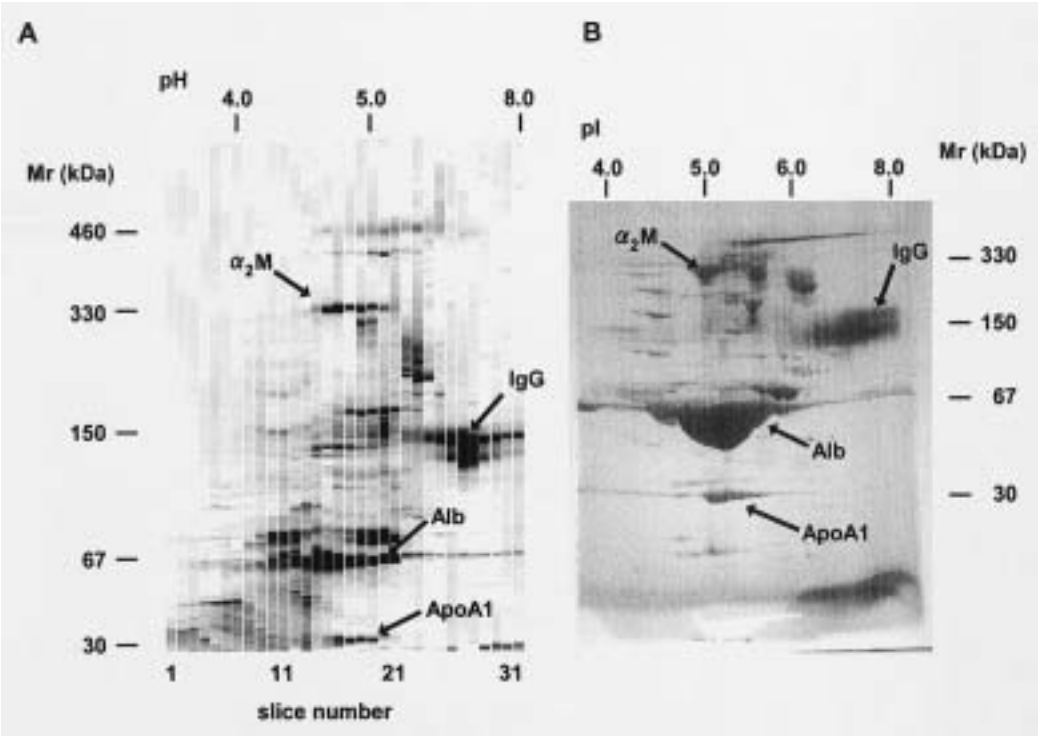

Figure 6. Comparisons of the 2−DE density pattern (A) with a 2−D gel electrophoresis pattern (B). The density pattern shown in Fig. 5 was rotated for 90 °anti−clockwise and the values in pI axis were obtained by running duplicated agarose IEF gels and measuring the pH in the gel slices and the values in Mr axis were obtained using a standard curve.

electrophoresis in the second dimension. Most of the spots in the 2−D density pattern were correlated with those on the 2−D gel electrophoresis pattern, suggesting that the SDS−CE patterns can be correlated with the protein spots in the 2−D gel electrophoresis pat-

tern. The narrower spot width in the direction of Mr axis in the density pattern (Figure 6 A) comes from higher resolution of SDS−CE technique [3]. These results demonstrated that when the protein peaks in SDS−CE patterns (*cf.* Figure 2 B, Figure 3, Figure 4 A) were further fractionated and analyzed [11], the information can be compared with that of protein spots on a 2−D gel electrophoresis pattern.

3.4.2 Comparison with performance of the 2−D separation method with 2−D gel electrophoresis

The efficiency of the present method is higher than the 2−D gel electrophoresis, since we could delete the manual steps of gel staining and destaining which normally require 2−3 days. Also, although we used a single−capillary apparatus, capillary array systems can be employed to aim higher efficiency in analysis [12, 13]. We have not tried to automate the steps of agarose IEF and protein extraction and the improvements on the efficiency of IEF−extraction steps awaits further investigation.

The sensitivity of the present method is higher than the 2−D gel electrophoresis method, since we used UV detection at 230 nm. We have estimated that the quantity of proteins injected in each CE run was about 1/3000 of that present in each IEF gel extract (data not shown). That means, if the IEF step can be directly combined with the SDS−CE step, the required volume of plasma can be calculated to be 1/3000 of the applied volume (50 μl), namely ca. 20 nl. This sample quantity is even smaller than that we need for micro 2−D PAGE, 1−2 μl.

For the direct identification of the proteins included in the CE peaks, the CE apparatus can be connected with a high resolution mass spectrometry. In fact, Fourier transform ion cyclotron resonance (FTICR) mass spectrometry is employed for the analysis of proteins separated by CE [14, 15] and it is claimed that this technique is much more efficient than the methods employed for the identification of proteins on a 2−D gel. We speculate that our 2−DE method could be combined with high−sensitivity MS for the identification of the separated proteins.

4 Conclusions

Two−dimensional separation of human plasma protein combining agarose gel IEF and SDS−CE showed a separation pattern which was similar to that of 2−D PAGE. This technique has obvious advantages in post−electrophoresis data processing when compared with conventional 2−D gel electrophoresis; protein quantity (as UV absorbance at 230 nm) can be obtained without staining, molecular masses can be estimated from the migration times of standard proteins, the separation results can be stored as digitized data.

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