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TWO-DIMENSIONAL SEPARATION SYSTEM FOR ANALYSIS OF PROTEINS EMPLOYING ISOELECTRIC FOCUSING AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A new type of separation system, which combined isoelectric focusing with high-performance liquid chromatography, was designed for the analysis and fractionation of serum proteins. For the first dimensional separation, carrier-free isoelectric focusing was used to separate proteins according to their electric charge. For the isoelectric focusing, an instrument which consisted of multiple chambers was deviced. For the second dimensional separation, high-performance gel permeation chromatography was used to separate proteins according to their molecular size. Human serum was subjected to analysis with this two-dimensional system, and separation of serum proteins according to their p*I* and molecular size was demonstrated.

INTRODUCTION

Two-dimensional separation methods, which combine two methods of different separation principles, are widely used for the separation of complex mixtures of proteins. In 1965, Moore and McGregor[1] separated soluble proteins from bovine brain by DEAE-cellulose chromatography, and each fraction was then subjected to starch gel electrophoresis. Two-dimensional electrophoretic techniques, employing polyacrylamide gel isoelectric focusing in the first dimension and polyacrylamide gradient gel electrophoresis[2] (or polyacrylamide slab gel electrophoresis in the presence of sodium dodecyl sulphate[3]) in the second dimension, offer the highest resolution of proteins at the present stage. However, when separation is performed employing starch gels or polyacrylamide gels as carriers, procedures for staining and destaining are necessary to detect proteins, and troublesome procedures are required to extract stained proteins from the gel matrix.

In this report, we describe a new type of separation system that combines carrier-free isoelectric focusing and high-performance gel permeation chromatography. In this system, proteins were separated by their electric charge in the first dimen-

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sion and by their molecular size in the second dimension. Applications of this system to the separation of human serum proteins are described.

EXPERIMENTAL

Reagents

Ampholine (pH 3.5–10, Lot No. 56) was obtained from LKB (Stockholm, Sweden). Other reagents were obtained from Wako (Osaka, Japan). Human albumin, transferrin, and immunoglobulin G (IgG) were industrially prepared in the Japanese Red Cross Central Blood Centre.

Preparation of serum samples

Fresh human blood (10 ml) was centrifuged at 800 *g* for 10 min. Sucrose was added to the serum up to 10% (w/v), and the serum was stored at −20°C and thawed at room temperature before use.

Apparatus

For the first dimension (carrier-free isoelectric focusing), an apparatus (side view shown in Fig. 1A) was constructed that consists of three parts: a column to be filled with cathode solution (Fig. 1A-a), a separation column to be filled with ampholine solution (Fig. 1A-b), and a column to be filled with anode solution (Fig. 1A-c); the total column length is 130 mm. The separation column consisted of seventeen acrylic unit plates with holes (4 mm diameter) (one of these is shown in Fig. 1B). These holes form a line during isoelectric focusing, and after isoelectric focusing the odd-numbered plates are shifted, so that the solution is separated in seventeen closed, small cells. The surfaces of the unit plates are coated with vaseline for water-sealing and then the unit plates are piled up. All parts are pressed together by nuts and bolts with springs inserted. The procedure for the fractionation of samples with this apparatus is shown in Fig. 2. Each fraction is taken out with a syringe (2-ml tuberculin syringe) equipped with a long needle (120 × 0.6 mm O.D.) and either injected into a chromatographic column or transferred to a small vial and stored in a refrigerator.

For the second dimension (gel permeation chromatography), a Model HLC-802 UR HPLC system (Toyo Soda, Yamaguchi, Japan) equipped with a UV spectrophotometer UVIDEC-100-II (JASCO, Tokyo, Japan) is used. For chromatography, a tandem column (two 600 × 7.5 mm stainless-steel columns) packed with G 3000 SW is used.

Isoelectric focusing

Carrier-free isoelectric focusing was performed as follows. On the bottom of the reservoir of the anode solution, a piece of dialysis membrane (3 cm × 3 cm) was attached and fixed with an O-ring. By a syringe equipped with a long needle, 0.75 ml of the anode solution (0.03 *M* phosphoric acid–40% sucrose) was poured into the anode reservoir (Fig. 1A-c), 0.43 ml of the ampholine solution (2% ampholine–20% sucrose) into the electrophoresis column (Fig. 1A-b), and 0.5 ml of the cathode solution (0.1 *M* sodium hydroxide) into the cathode reservoir (Fig. 1A-a), in that order. Sample solution (10 μl) was added at the top of the ampholine solution with a microsyringe. Then the apparatus was put in a polyethylene vessel (120 mm × 80 mm

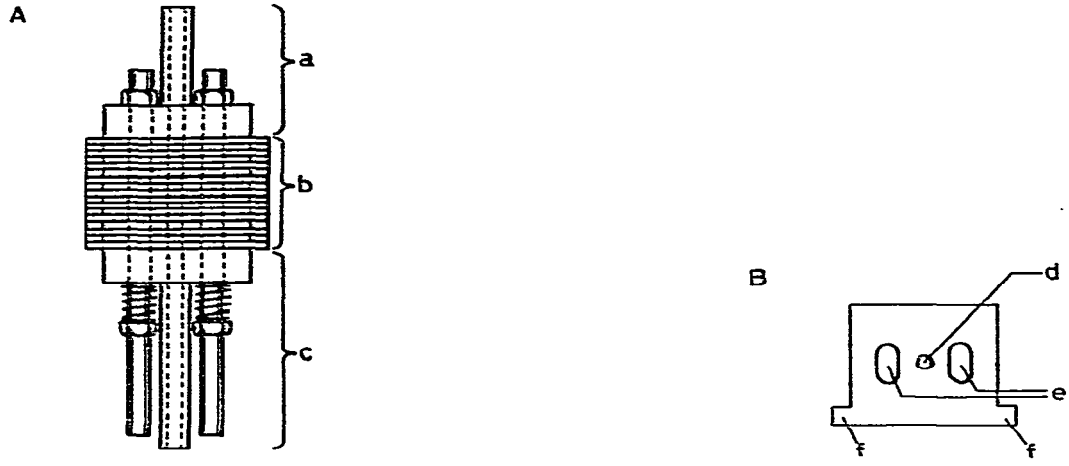

Fig. 1. Carrier-free isoelectric focusing apparatus. (A) Side view of the apparatus: a, column to be filled with cathode solution; b, column to be filled with 2% ampholine solution; c, column to be filled with anode solution; the total column length is 130 mm. B, Top view of a unit plate of column b (50 × 40 × 2 mm): d, hole (all holes together form the electrophoretic column for isoelectric focusing); e, holes for bolts; f, notches for shifting the unit plate.

I.D.) and the vessel was filled with the anode solution so that the electrophoretic column was completely dipped in the anode solution. The vessel was cooled with ice-water. Isoelectric focusing was run at a constant current of 2 mA for 20 min and then at a constant voltage of 460 V for 17 h.

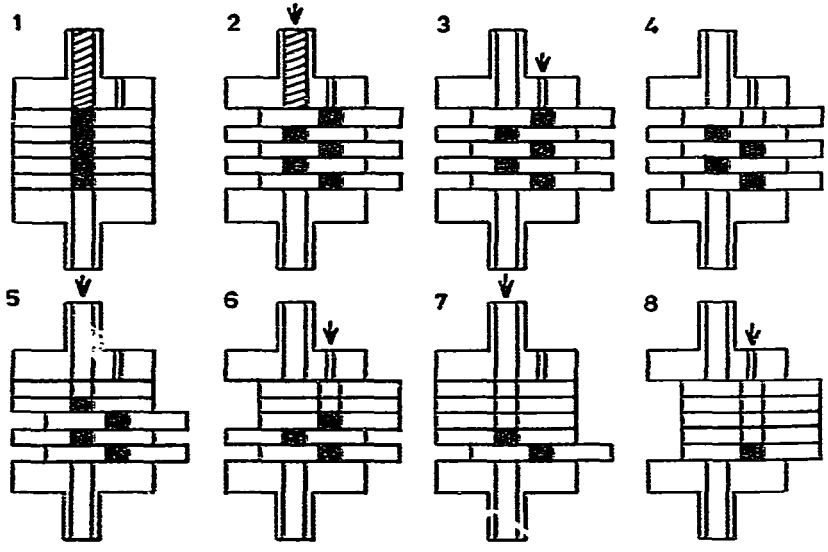

Fig. 2. Method for the fractionation of protein bands after isoelectric focusing. (1) Position of unit plates during isoelectric focusing. (2) Position of unit plates after isoelectric focusing. Odd-numbered unit plates were shifted to fractionate protein bands. The needle of a syringe was inserted as shown by an arrow to take out the solution in the top cell. (3–8) The process of taking the solution out of the cells.

High-performance liquid chromatography

After isoelectric focusing, each fraction was subjected to high-performance gel permeation chromatography. A 25-μl volume of each fraction was introduced into the column and developed with an elution buffer (0.05 *M* sodium acetate containing 0.2 *M* sodium sulphate, pH 5.0). The flow-rate was 1 ml/min at room temperature. The absorbance of the eluate was monitored at 230 nm. The time necessary for the separation of one sample was *ca.* 50 min.

RESULTS

Separation of serum proteins by carrier-free isoelectric focusing

Fig. 3 shows an example of the separation of a serum sample. The pH values and the protein contents in the seventeen fractions obtained by the carrier-free isoelectric focusing apparatus are shown. The pH range of the fractions was from 7.08 (fraction 1) to 4.90 (fraction 17). Protein content is shown as a percentage of the total protein.

Separation of IEF-fractionated serum proteins by HPLC system

Fig. 4 shows HPLC elution patterns of the IEF fractions 1–10. Fraction 1 was the most alkaline fraction (pH 7.1). Arrows under the elution patterns show the peak positions of purified proteins.

As shown in the elution patterns of fractions 1–3 (the pH values of the three fractions were 7.1, 6.8 and 6.7 respectively), IgG in these fractions showed a symmetrical peak, suggesting the absence of other protein species in the peak. This implies that IgG in a serum sample could be purified by this IEF–HPLC system. Fraction 10 also had a peak close to the peak position of IgG. However, this peak should represent a protein species other than IgG, because fraction 10 contained proteins of p*I* 6.0, which is outside the p*I* range of IgG. Seventy-five peaks were counted in the seventeen elution patterns of a serum sample, excluding the peaks of ampholine.

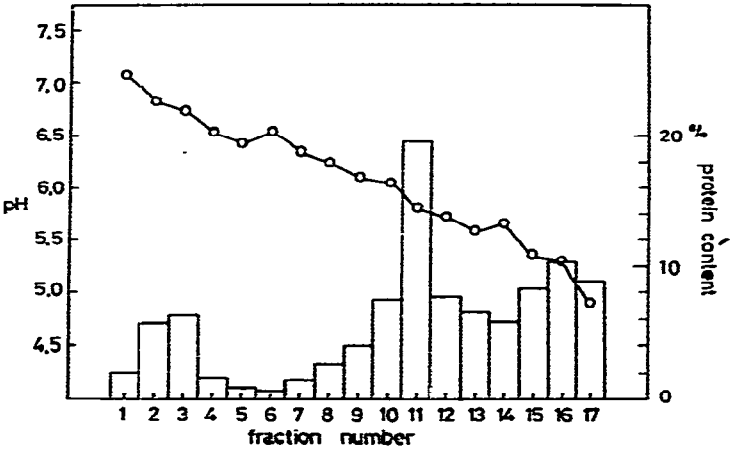

Fig. 3. Separation of human proteins with the carrier-free isoelectric focusing apparatus. The pH gradient and the protein contents (bars, % of total protein) of the seventeen fractions are shown. The most alkaline fraction was fraction 1 and the most acidic one was fraction 17.

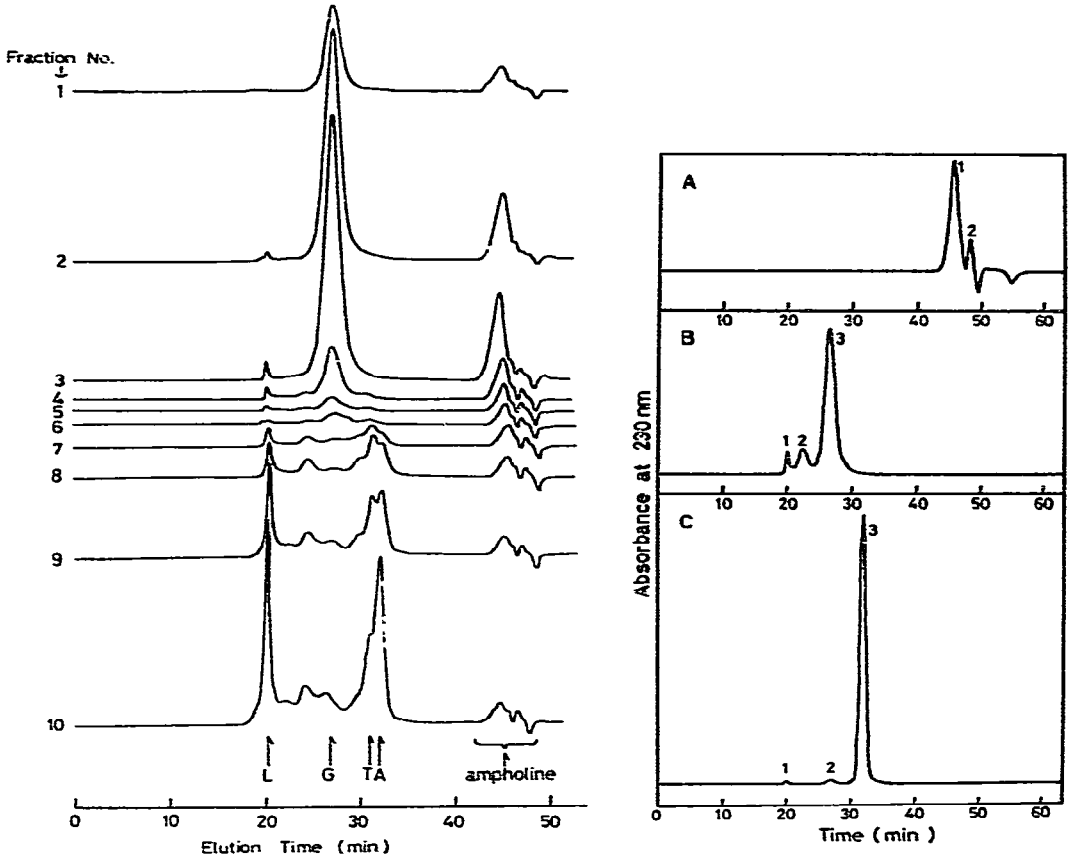

Fig. 4. High-performance gel permeation chromatograms of fractions (fractions 1–10) obtained by the carrier-free isoelectric focusing apparatus. Buffer, 0.05 *M* sodium acetate containing 0.2 *M* sodium sulphate, pH 5.0; flow-rate, 1 ml/min; detection, UV (230 nm). Arrows indicate the elution time of: L, lipoprotein; G, IgG; T, transferrin; A, albumin.

Fig. 5. Elution patterns of ampholine and purified proteins. (A) 2% Ampholine (pH 3.5–10)–20% sucrose, 20 μl. (B) Human serum IgG (0.15%), 20 μl. (C) Human serum albumin (0.2%), 20 μl. Buffer, 0.05 *M* sodium acetate containing 0.2 *M* sodium sulphate, pH 5.0; flow-rate, 1 ml/min; detection, UV (230 nm).

Elutions of purified serum proteins and ampholine by HPLC

Fig. 5A shows an elution pattern of ampholine by HPLC. When 20 μl of 2% ampholine (pH 3.5–10)–20% sucrose was applied, two peaks appeared (1 and 2 in Fig. 5A) and their elution times were between 40 min and 50 min.

Fig. 5B shows an elution pattern of human serum IgG (0.15%, 20 μl). Peaks 3, 2, and 1 were identified from a molecular weight standard curve[4] to be monomer, dimer, and other polymers of IgG, respectively. Elution time of the monomer was 27 min.

Fig. 5C shows an elution pattern of human serum albumin (0.2%, 20 μl). Peak 3, 2, and 1 were identified to be albumin monomer, dimer, and polymers, respectively. Elution time of the monomer was 32 min.

DISCUSSION

Two-dimensional separation is a feasible method for analysis of complex mixtures of proteins such as serum proteins. Various combinations of different techniques have been used for two-dimensional separation[5–7]. Combination of carrier-free isoelectric focusing and high-performance gel permeation chromatography was chosen for the following reasons. (1) This system separates proteins by two independent factors, p*I* in the carrier-free isoelectric focusing and molecular weight in the high-performance gel permeation chromatography. The p*I* values in each fraction obtained by carrier-free isoelectric focusing and the molecular weight of proteins separated by HPLC can be measured. Thus, proteins separated by this system can be assigned by p*I* and molecular weight. (2) Proteins can be separated without using a gel matrix. Therefore, no troublesome procedures to extract proteins from the gel matrix are necessary, and the processes of staining and destaining are not required to detect proteins because they can be detected by UV absorbance.

Automatic separation will become easy in this system because proteins are separated in solution and no operation to extract proteins from the gel matrix is necessary. Construction of an automatic sample take-out device is in progress.

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