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GEL PERMEATION CHROMATOGRAPHY COMBINED WITH CAPILLARY ELECTROPHORESIS FOR MICROANALYSIS OF PROTEINS

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SUMMARY

A new type of apparatus for the analysis of complex protein mixtures, in which gel permeation chromatography was combined with capillary electrophoresis, was constructed. Proteins were separated according to their molecular size in the first step, then separated according to their electrophoretic mobility in the second step. The outlet of a microbore column was connected with the sample injection port of a capillary electrophoresis apparatus. All the procedures of chromatography and electrophoresis were automated with the aid of a system controller. Preliminary results on the separation of proteins are presented.

INTRODUCTION

Two-step separation methods, which combine two methods based on different separation principles, are widely used for the analysis of complex protein mixtures[1–3]. Two-dimensional electrophoresis is one such method, employing polyacrylamide gel isoelectric focusing in the first and polyacrylamide slab gel electrophoresis in the second dimension[4,5], and offers the highest resolution of proteins at present. However, electrophoretic techniques which employ polyacrylamide gels have the disadvantages that the preparation of gels is a complicated and time-consuming procedure and proteins can be detected or determined only after they have been fixed and stained on the gel supports. Because of the complex procedures and difficulties in quantification, automation of two-dimensional polyacrylamide gel electrophoresis has not been achieved.

In this paper, we describe a new type of separation system which combines gel permeation chromatography with capillary electrophoresis (isotachophoresis) for the analysis of proteins. As neither method includes the gel preparation or protein fixation steps, all the procedures could be automated.

EXPERIMENTAL

Apparatus

A schematic diagram of the combined apparatus is shown in Fig. 1. A micro-

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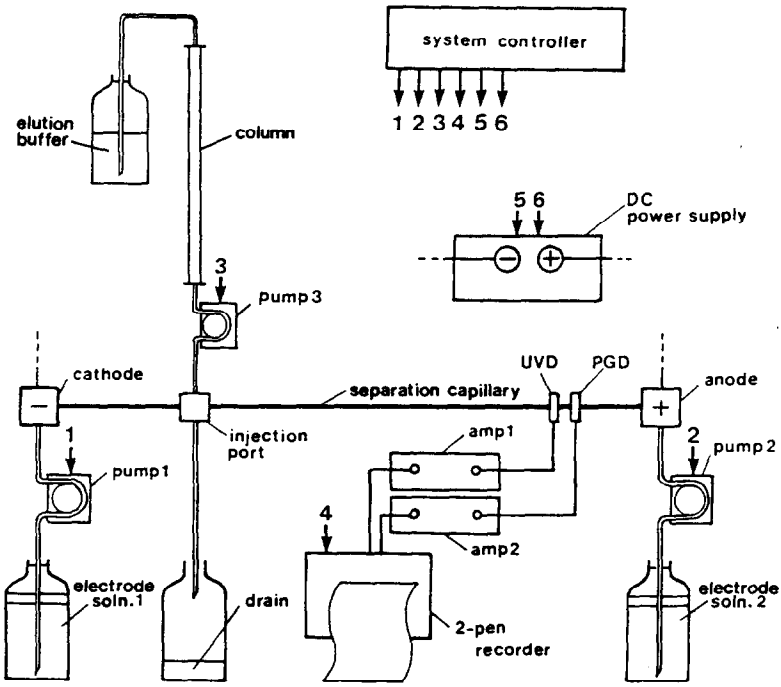

Fig. 1. Combined chromatographic and capillary electrophoretic apparatus. The terminating electrolyte (solution 1) and the leading electrolyte (solution 2) are pumped by peristaltic pumps (pumps 1 and 2) to wash the electrodes and separation capillary. The effluent from the column is loaded with the microperistaltic pump (pump 3) through the sample injection port. A d.c. high voltage is applied between the electrodes and the protein zones are detected with the potential gradient and UV detectors during the run. The numbered arrows indicate the output lines connecting the system controller to the equipment.

bore chromatographic column made from polyethylene tubing (270 mm × 2.3 mm O.D. × 1.3 mm I.D.), packed with Sephadex G-50 fine gel permeation support (Pharmacia, Uppsala, Sweden), was set adjacent to the fully automated apparatus for capillary electrophoresis[6]. A microperistaltic pump (Chromato Research, Yokohama, Japan) was employed to introduce the column effluent into the sample injection port of the electrophoresis apparatus. Perfluorinated ethylene–propylene (PFEP) tubing (50 mm × 0.5 mm I.D.) was used to connect the column outlet to the silicone-rubber tubing (50 mm × 5 mm O.D. × 1 mm I.D.) of the microperistaltic pump. Another PFEP tube (50 mm × 0.5 mm I.D.) was used to transfer the column effluent from the silicone-rubber tubing of the pump to a glass capillary tube (50 mm × 0.35 mm O.D. × 0.24 mm I.D.) that had been inserted in the injection port of the electrophoresis apparatus. The apparatus for capillary electrophoresis is basically the same as that reported previously[6], except the automatic sampler has been removed.

System controller

For the automatic operation of the combined apparatus, the microperistaltic pump of the chromatographic system was also controlled by the system controller of

the capillary electrophoresis apparatus. Details of the construction of the system controller have been fully described elsewhere[6]. The system controller consisted of an 8-bit microcomputer (NEC PC-8001; Nippon Electric, Tokyo, Japan) equipped with an FGU-8000 640 dot × 200 dot graphic unit, an input–output (I/O) unit (NEC PC-8013; Nippon Electric), a relay interface board, a 5-in. floppy disk unit (NEC PC-8031; Nippon Electric) and a dot-matrix printer (MP-82, Epson, Nagano, Japan).

Computer programs

The microcomputer programs for system control were written in BASIC. The time schedule of the program is described under Results.

Materials

Bovine serum albumin was obtained from Nutritional Biochemicals (Cleveland, OH, U.S.A.), myoglobin (from horse heart) from Sigma (St. Louis, MO, U.S.A.) and tyrosine from Ajinomoto (Tokyo, Japan). An ampholytes mixture (Ampholine pH 3.5-10) was obtained from LKB (Bromma, Sweden). 2-Amino-2-methyl-1-propanol was obtained from Nakarai Chemicals (Kyoto, Japan), tranexamic acid from Daiichi Seiyaku (Tokyo, Japan), hydrochloric acid (1 *M*, special grade for amino acid sequence analysis), potassium hydroxide and hexane from Wako (Osaka, Japan), hydroxypropylmethylcellulose (HPMC) from Aldrich (Milwaukee, WI, U.S.A.) and sodium azide from Kanto Pure Chemical (Tokyo, Japan). Reagents for the preparation of the electrode solutions were used without further purification.

Gel permeation chromatography followed by capillary electrophoresis

A standard sample solution was prepared as follows. Aliquots of protein solutions, albumin (50 mg/ml, 5 μl), myoglobin (5 mg/ml, 10 μl) and tyrosine (1 mg/ml, 5 μl), were mixed and a 5 μl portion was applied to the column. The elution buffer for gel permeation chromatography was 0.25% Ampholine (pH 3.5 10)–0.00625% sodium azide. The conditions for capillary electrophoresis were as follows. The leading electrolyte solution was 5 m*M* HCl–9.3 m*M* 2-amino-2-methyl-1-propanol (pH 9.9). The terminating electrolyte solution was 50 m*M* tranexamic acid–potassium hydroxide (pH 10.8). The solutions were kept in amber-glass bottles and overlayed with a 1-cm layer of hexane to minimize the dissolution of carbon dioxide. A PFEP capillary tube (230 mm × 0.5 mm I.D. × 1.0 mm O.D.) was used as the separation tube, the inner surface of which had been coated with HPMC[6]. In each cycle of the automatic analysis, the electrolyte solutions were pumped to wash the electrode and the separation tube. The volumes of the leading and terminating solutions pumped were 2 and 0.75 ml in 2 and 3 min, respectively. The column effluent was loaded in the injection port by the microperistaltic pump, 9 μl in 6 s. Electrophoresis was run at a constant current of 150 μA for 4.8 min (initial voltage about 6 kV) and then at a constant current of 50 μA for 7.8 min.

RESULTS

Gel permeation chromatography

Before combination with the electrophoresis apparatus, gel permeation chromatography was performed separately. Fig. 2 shows the elution profile of the stan-

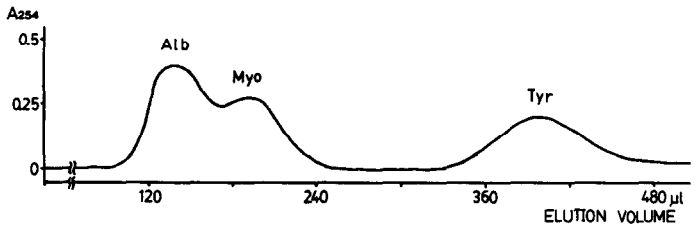

Fig. 2. Elution profile of proteins and an amino acid by gel permeation chromatography. The sample solution (albumin 62 µg, myoglobin 12 µg and tyrosine 1.2 µg in 5 µl) was applied on to the polyethylene column (270 mm × 1.3 mm I.D.) and eluted with the elution buffer (0.25% Ampholine–0.00625% sodium azide) at a flow-rate of 114 µl/h. Alb = Bovine serum albumin; Myo = myoglobin; Tyr = tyrosine.

dard samples. The sample solution (5 µl) was applied to the column and eluted at a flow rate of 114 µl/h (8.8 ml/cm^2·h). As can be seen, myoglobin appeared as a shoulder after the peak of albumin.

Combined apparatus and time schedule for automated operation

The chromatographic system was combined with the capillary electrophoresis apparatus as described under Experimental (Fig. 1). The column effluent was introduced directly into the injection port of the electrophoresis apparatus by the microperistaltic pump (sampling pump). For automatic operation of the combined apparatus, all the equipment, the sampling pump, the peristaltic pumps for loading of the electrode solutions, a recorder and a high-voltage d.c./power supply, were controlled by the system controller. The time schedule is shown in Fig. 3: (1) pump the leading electrolyte solution to rinse the leading electrode and the separation capillary; (2) pump the terminating electrolyte solution to rinse the terminating electrode; (3) pump the effluent from the column to the injection port; (4) turn on the d.c./power supply to start electrophoresis (150 µA constant current); (5) turn on the two-pen recorder and reduce the current to 50 µA; (6) turn off the d.c./power supply and the recorder. Then these procedures were repeated from step 1. The time needed for one cycle of analysis was 18 min.

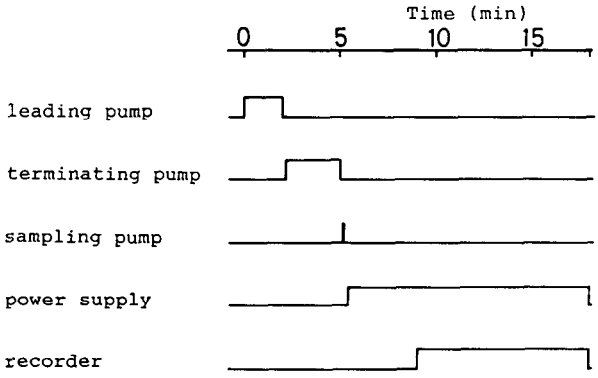

Fig. 3. Time programme for automated analysis of the samples. The length of the upper steps in the time course represents the "on" time of each equipment. The time for one analysis was 18 min.

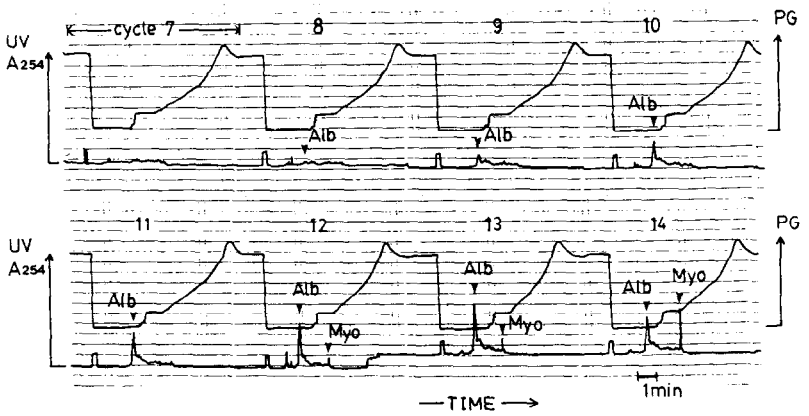

Fig. 4. Electropherograms obtained from the sequential analysis of the column effluent. The sample solution (5 μl) was applied to the combined apparatus. Eight electropherograms from the 7th to 14th cycles are shown. The upper and the lower curves show the potential gradient (PG) and UV absorbance, respectively.

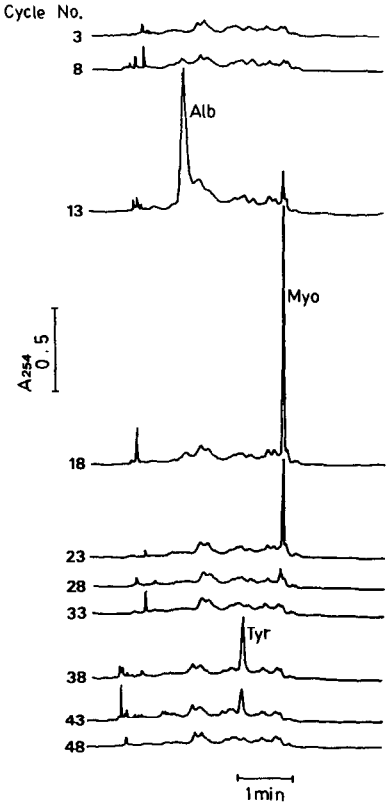

Fig. 5. Separation of albumin, myoglobin and tyrosine with the combined apparatus. The UV patterns of the electropherograms were traced every five cycles and the separation of the samples was demonstrated.

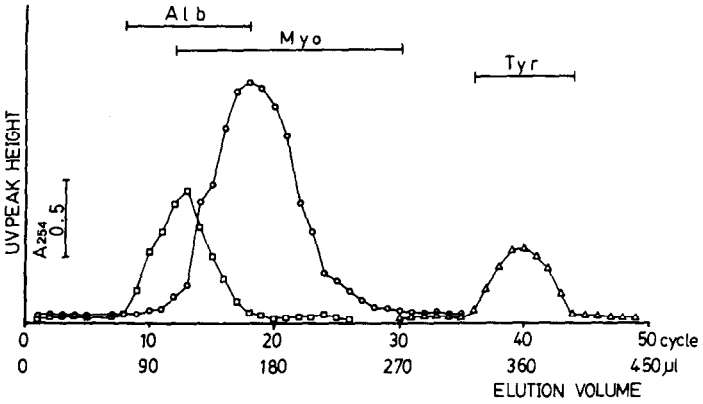

Fig. 6. Traces of the UV peak height obtained from the sequential analysis. The UV peak height of the samples in the electropherograms obtained sequentially was plotted against the cycle number. In comparison with the results of one-dimensional separation (Fig. 2), an improved sensitivity of the samples and the resolution of albumin and myoglobin as two separate peaks were demonstrated.

Sequential analysis of column effluents

The sample solution (5 µl) was applied to the column of the combined apparatus. The column volume was 365 µl, the line dead volume was calculated to be 59 µl and the volume of the effluent pumped in one cycle was set at 9 µl. Then the cycle described above was repeated 60 times within 18 h, without manual operation. For convenience in calculating the elution volume, the initial seven cycles were not counted. Some of the electropherograms sequentially obtained are shown in Fig. 4. The upper and lower curves show the potential gradient value and UV absorbance, respectively. Although the analysis time in one cycle was 18 min, only the part for the last 9 min was recorded (as shown by the time schedule in Fig. 3). As judged from the shape of the potential gradient curves, the electrophoretic runs were fairly reproducible. A UV peak that appeared in the electropherogram of the eighth cycle and had a maximum height at the thirteenth cycle was identified to be that of albumin from the peak position. Likewise, the peak of myoglobin was identified in the 12–14th electropherograms.

The electropherograms were traced every five cycles (Fig. 5). The applied samples, albumin, myoglobin and tyrosine, appeared as sharp UV peaks. Each sample was separated with a characteristic electrophoretic mobility. Further, the samples were concentrated as narrow zones in the process of isotachophoretic separation and were detected at elevated sensitivity compared with direct UV measurement of the chromatographic effluent (Fig. 2).

The UV peak heights of the samples that were measured from the electropherograms were plotted against the analysis cycle number (Fig. 6). By the combined analysis, the pattern of gel permeation chromatograpy could be re-traced with the information on the amount of the constituent proteins. Further, the samples could be detected with more than twice the sensitivity.

DISCUSSION

We have been attempting to construct systems for the high-resolution analysis of proteins in mammalian body fluids and tissues[4,7]. For the analysis of proteins in these complex protein mixtures, two-step separation techniques based on two different principles are required. Two-dimensional polyacrylamide gel electrophoresis offers the highest resolution of proteins at present, but it has the disadvantage of troublesome operational procedures. In order to simplify the operation, we have constructed a two-step separation system in which proteins are separated in solution[8]. With this system, proteins could be fractionated without extraction from the gel matrix. The two-step system reported here was constructed to analyse proteins with high sensitivity and simple operation. The combination of gel permeation chromatography and capillary electrophoresis (isotachophoresis) was established for the following reasons: proteins can be separated on the basis of two independent parameters, molecular weight and electrophoretic mobility; and proteins diluted in the gel permeation chromatographic step can be concentrated in the subsequent isotachophoretic step[9]. As shown under Results, proteins were separated in solution and all the procedures were automated. The minimum detectable amount of proteins in this system was about 200 ng, which depended on the sensitivity of the capillary isotachophoretic step. This level of sensitivity is comparable to that of polyacrylamide gel electrophoresis, for which automated apparatus has not been reported.

For the first step of separation by gel permeation chromatography, a microbore open column and a microperistaltic pump were employed to avoid electric leakage. However, for better resolution of proteins in this first step, a gel permeation high-performance liquid chromatographic (HPLC) column and a high-pressure pump could be used. The construction of an apparatus with an HPLC system is in progress.

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