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SEPARATION OF PROTEINS IN A TRANSIENT PH GRADIENT COLUMN

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Abstract—Mixtures of four proteins, sperm whale myoglobin, horse myoglobin, transferrin and albumin of isoelectric points, 8.3, 6.8-7.8, 5.9, 4.7 respectively, were successfully separated by chromatofocusing in a column packed with PBE 94 under transient pH gradients. Eluting the polybuffer, the smooth and linear pH gradient was developed for PBE 94 exchanger, and each of four proteins was recovered in the order of PI's with high recovery yields, and within 0.25 pH unit from the elution pH's. A simulated mixture of four proteins was separated out at the corresponding pH. The internal elution pattern of Myoglobin I was visually determined in the gradient column and the focusing effect of protein by pH was illustrated with the elution of intermittent feeds into a single band.

INTRODUCTION

Among the various methods of protein separation, chromatofocusing has been introduced to be one of the most distinctive techniques combining the high resolving power of isoelectric focusing and the convenience of chromatography [1-3]. After the successful application of Sluyterman et al. [4-7] the strong focusing effects at the isoelectric points of proteins, generated by the chemically-defined internal pH gradient on an ion exchange column, have been reported for a number of biologically active fluids [8-20]. In chromatographic techniques having a focusing effect, there are several advantages of rapid separation, easy operation and column selection [9,10], and no cooling problems like electrophoresis [8-10]. In addition, proteins which could not be separated by isoelectric focusing, were separated analytically [11,12] or in a preparative scale [9-16].

Surface charges of proteins can be regulated by proteins or counter ions adsorbed on the surface [17]. When the surface charge reaches zero as shown in Figure 1 (zero point of charge, called isoelectric point, PI), the pH is the isoelectric point of protein, and zeta-potential falls to zero. In chromatofocusing column packed with an anion exchanger, proteins would be concentrated at bands near the pH's of their isoelectric points. Since the linear gradient of pH will be most appreciated for the good resolution and reproducibility, the buffers with a constant buffering capacity at a wide pH range are required [5,6]. In spite of commercial products, however, the complex internal mechanisms are not elucidated so as to include all the physical interactions [4].

According to Sluyterman et al. [4,5], the pH variation can be calculated by mixing a buffer of flowing through gels and a stagnant buffer between gels, and by incorporating the buffering action of buffers [4,5]. When equal volumes of aliquots of both phases are mixed, the pH can be obtained from the amounts of the moving phase and the stationary phase, and from the buffering capacities.

In this study, an experimental simulation was performed in order to investigate the protein separation and the chromatofocusing effect in a transient pH gradient column. The transient internal pH gradients were generated by flowing an elution buffer through the gels maintained at a certain pH, and four proteins were separated. The chromatofocusing effect of proteins for sequential feeds was also investigated for a preparative purpose.

EXPERIMENTS

Chemicals

Four proteins of myoglobin I and II, transferrin and albumin and all buffering compounds have been purchased from Sigma Chemical Co. and Bio-Rad Labora-

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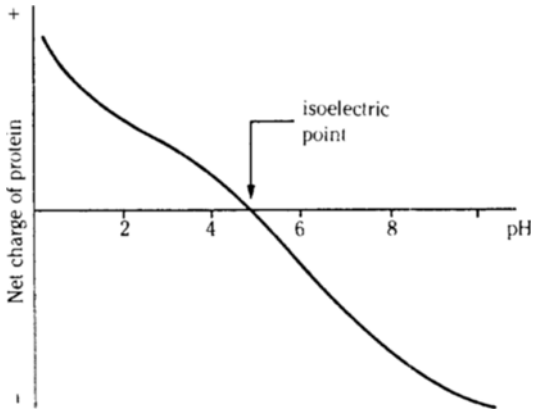


Fig. 1. The net charge on a model protein as a function of pH.

tories, and used without further purifications. Molecular weights and PI's of proteins were reported in Table 1 [5,18]. The DEAE Bio-Gel A for ion exchanger column was purchased from Bio-Rad Laboratories, and Polybuffer 74 and Polybuffer 96 and ion exchanger PBE 94 from Pharmacia Fine Chemiccals [19].

The aqueous solutions of hydrogen chloride and sodium hydroxide, 0.1 mole and 1 mole, were formulated with doubly distilled water in our laboratory. The buffering capacities of buffers were titrated with hydrogen chloride or sodium hydroxide and a good linearity in the range of experiments was obtained and determined with the acid solution of hydrogen chloride by the electric conductivity and pH [20].

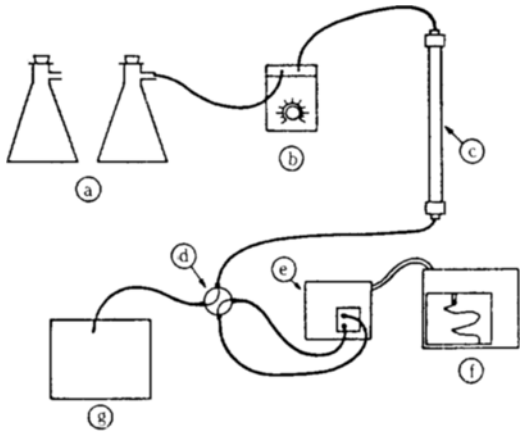
Equipments and Method

A schematic diagram of the equipments was given in Figure 2. A chromatographic column of Type C 10/50 of Pharmacia Fine Chemicals and a heavy wall pyrex tube of outside diameter 12 mm and inside diameter 10 mm were installed vertically. The internal of the column was packed with swollen beads of DEAE Bio-Gel A or PBE 94.

After gels were degassed for 5 to 10 hours, the equilibrium buffer flowed through the gel bed at the rate of 0.3 to 0.8 ml/min. The elution buffer was pumped with EYELA MicroTube Pump MP-3 (Tokyo Rikakikai

Table 1. Molecular weights and isoelectric points (PI's) of proteins

	Molecular weight	PI	Remark
Albumin	65,000	4.7	[18]
Myoglobin I	17,800	8.3	sperm whale [5]
Myoglobin II	16,600	6.8-7.8	horse [18]
Transferrin	77,000	5.9	[18]



a : buffer reservoir
b : peristaltic pump
c : column
d : 4-way valve
e : UV-1 monitor
f : recorder
g : Auto-Sampler

Fig. 2. Schematic diagram of equipments.

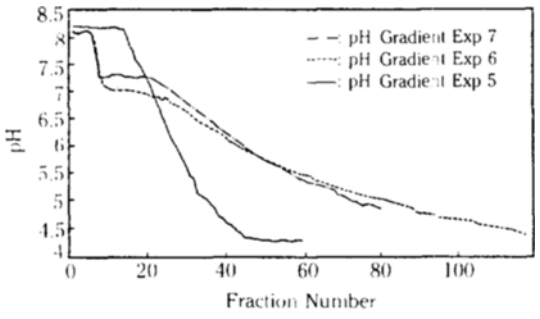
Co., Ltd) through a silicon tube and elution aliquots were collected continuously by Auto-Sampler (Technicon, Ins. Co.). When elution volumes reached 10 to 15 bed volumes, its original pH and electric conductivity, it was approximated that gel was equilibrated with the equilibrium buffer. Then the elution buffer started to elute through the equilibrated column to produce a linear pH gradient and then the protein solutions of the elution buffer were injected.

The concentrations of proteins were determined by Single Path Monitor UV-1 (Pharmacia Fine Chemicals) at 280 nm and recorded with Gow-Mac Model 70-750 Recorder (Gow-Mac Ins. Co.). The pH was determined by Dongwoo Medical DP-215 pH meter with Beckman 39836 Combination electrode and the electric conductivity by YSI model 31 conductivity bridge with 3404 cell (Yellow Springs Instruments Co., Inc.). The data has been filed on a personal computer. The internal peaks of protein, myoglobin I having a dark brown, were determined visually and by taking pictures.

RESULTS AND DISCUSSIONS

pH gradients

The ion exchanger consisting of a cross-linked agarose support is very stable in the variations of pH and ionic strength. The gel structure was specially designed for free access of protein such as the polyethyleneimine branch for PBE 94 [15]. The gel in the column had no change in volume when it was stabilized. The effective liquid volume in the mobile phase was 70 to 80 % of the bed volume [19]. Since the shape of the pH gradient is important in column chromatography



• Experimental Condition

	Exp. 5	Exp. 6	Exp. 7
Ion Exchanger	DEAE Bio-Gel A	PBE 94	PBE 94
Column Length	23.5 cm	19.6 cm	19.6 cm
Flow Rate		1.0 ml/min	
Equilibration Buffer	0.02 M Tris-HCl pH 8.20	0.05 M Tris-HCl pH 8.20	0.02 M Tri-HCl pH 8.20
Elution Buffer		10 %(v/v) PB 74-HCl pH 4.49	

Fig. 3. Variations of pH gradient in the column of DEAE Bio-Gel A and PBE 94.

for resolution power and separation time, the simulation of the gradient was performed experimentally. In Figure 3 of the PBE 94 and DEAE Bio-Gel A column, the smooth and reproducible gradients of pH were achieved in the wide range of pH 9 to 4, when Tris (or Ethanolamine) was used as the equilibrium buffer and the Polybuffer as the elution buffer. The increase of the concentration of elution buffer has been reported to in-

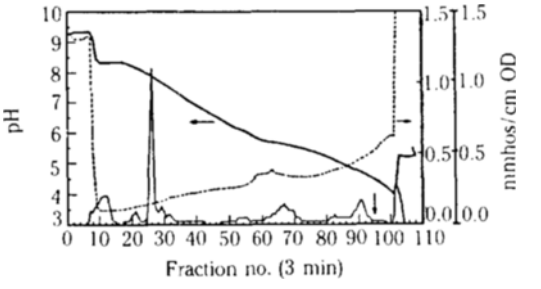


Fig. 4. Elution profile of pH, electrical conductivity, and optical density of myoglobin type I from the column packed with PBE 94.

The bed volume, 23.6 ml packed with PBE 94 in C 10/50 column of Pharmacia. The equilibration buffer was 0.025 M ethanolamine-HCl, pH 8.6 and the elution buffer in the rate of 0.6 ml/min was 10 %(v/v) PB 74/PB 96 (7:3)-HCl, pH 5.74. The (-●-●-) for optical density, the (———) for the pH of the eluent and the (----------) for electric conductivity, mmhos/cm. The vertical arrow indicated the start of 1M NaCl washing.

Table 2. Elution of single protein in PBE 94 packed column

protein	PI (observed)	pH slope (pH/ml)	band ΔpH	width ΔV ml	yield %
Myoglobin I	7.90	0.008	0.03	6	73
Myoglobin II	8.55	0.012	0.07	6	72
Albumin	4.50	0.009	0.09	19	81

crease the gradient of pH [5,16]. In our experiments, further, the increase of the concentration of equilibrium buffer decreased the slope of gradients but the effect was smaller than that of the elution buffer. The increase of the flow rate of the medium and the length of the column reduced the gradient [5,6]. DEAE Bio-Gel A made larger gradient than PBE 94, because the buffering capacity of DEAE Bio-Gel A has only 1/5 of PBE 94's, which is consistent with the observations of Sluytermann et al. [6].

Protein separation

The individual PI values were determined from the pure samples as purchased and consistent with the reported within 0.25 pH unit [5,18]. Since purchased proteins contained some impurities, several unknown peaks have been detected (Figure 4) and the yields of proteins were approximately over 70% in Table 2 [20]. This is because there are some impurities and because the strong binding of protein which can not be desorbed.

In Figure 5, the elution profile of an example run of an artificial mixture of four proteins, myoglobin I and II, transferrin and albumin with isoelectric points of 8.3, 6.8-7.8, 5.9, 4.7 respectively was shown. The mixed sample was prepared in 8 ml of equilibration buffer

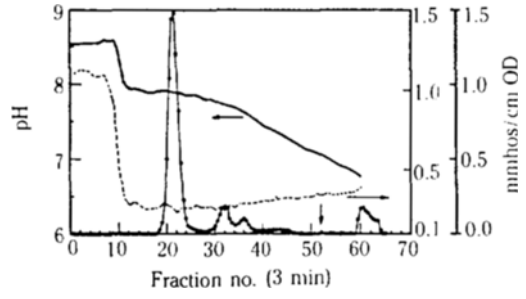


Fig. 5. Elution profile of pH, electric conductivity and optical density of protein mixtures of myoglobin I and II, transferrin, and albumin from the column packed with PBE 94.

The mixed sample was prepared in 8 ml of equilibration buffer by mixing 4 mg of each protein and the experimental conditions were given in Figure 4 equilibration buffer was pH 9.3 and elution buffer was pH 3.94.

Table 3. Separation of protein mixtures in PBE 94 packed column

No.	protein	PI (observed)	pH slope (pH/ml)	band ΔpH	width ΔVml	yield %
1.	Myoglobin I	7.84	0.016	0.06	3	88
	Myoglobin II	8.34	0.016	0.12	17	65
	Albumin	4.73	0.016	0.11	10	90
	Transferrin	5.64	0.016	0.15	21	98
2.	Myoglobin I	7.89	0.012	0.03	3	60
	Myoglobin II	8.79	0.012	0.01	5	84
	Albumin	4.62	0.012	0.18	10	82
	Transferrin	5.80	0.012	0.17	14	88
3.	Myoglobin I	7.69	0.013	0.04	8	73
	Myoglobin II	8.20	0.013	0.01	6	100
	Albumin	4.70	0.013	0.17	10	45
	Transferrin	5.65	0.013	0.20	23	93

by mixing 4 mg of each protein and the bed volume, 23.6 ml packed with PBE 94 in C 10/50 column of Pharmacia. The equilibration buffer was 0.025 M ethanolamine-HCl, pH 9.3 and the elution buffer in the rate of 0.6 ml/min was 10% (v/v) PB 74/PB 96 (7:3)-HCl, pH 3.94. The narrow solid line represents the concentration chromatogram shown in optical density, the solid line for the pH of the eluent and the dotted for electric conductivity, mmhos/cm. The vertical arrow indicated the start of 1M NaCl washing. The reproducibility of the protein elution was reported in Table 3.

Whether protein samples were introduced or not, the electrical conductivities showed a large irregularity in an early stage of elutions and after one bed volume of elution, a rapid drop was observed and then smoothed out having a minimum at the range of one space time. Here, ions affecting the conductivities of the media may be proton and Cl^-. However, since proton combines with a buffer molecule at this pH, the concentration of proton decreases. In fact, the proton concentration of 16 mM HCl in the buffer solution appeared to be near zero, and the active ions affecting the behavior of conductivity curves will be chloride ions. The small increase of conductivity as pH decreases, may be attributed from the proton but the contribution from the other mobile ions is not significant. Therefore, proteins are mainly separated by pH rather than the ionic strength.

Chromatofocusing Effects

In Figure 6, the flow profiles of myoglobin I in the column internal were shown at the sequential feeds by taking pictures. After the first sample flowing down to a certain point, the second sample with the identical protein was eluted. As flow advanced, the main peaks

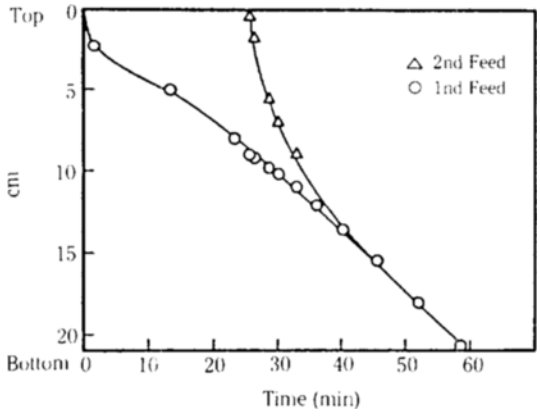


Fig. 6. The chromatofocusing effect of myoglobin I in the column packed with PBE 94.
The column length 21 cm, the equilibrium buffer, 0.025 M ethanol amine-CH_3COOH, pH 9.27 and the elution buffer at the flow rates of 0.6 ml/min was PB 96(10 %v/v), pH 5.97. The protein samples, 0.3 ml were fed at an interval of 26 min and the concentration peaks were determined by taking pictures.

of the solutions merged at the location where the transient pH was equivalent to the PI and the proteins flow down together until eluted. This experiment indicated that protein flows fast when the difference (pH-PI) is negative, and that the protein can be accumulated into a band at the pH until eluted. This phenomena may be indicative for the preparative purpose of proteins.

Similar to the ion exchange column [21], the transient pH gradient in the moving phase or the stationary phase is determined from the axial diffusion, the bulk mixing, and the regulations of proton by the gel and buffer interaction. Assuming the proton generation by the adsorption-desorption at an interface and by the buffer action, the transient gradient of the moving phase can be obtained by,

$$\frac{\partial c_i}{\partial t} = \frac{\partial c_i}{\partial t}\Big|_{\substack{ion\\exchange\\column}} + \frac{\partial c_i}{\partial t}\Big|_{\substack{buffer\\action}} \qquad (1)$$

where the c_i is the concentration of proton at the mobile phase. The rate of proton change at ion exchange column, includes the axial dispersion of proton, the bulk convective flow, and the generation of hydrogen ion from the stationary phase, and that of buffering action from the regulation of proton by buffering molecules. For protein elutions, the mechanism of ion exchanger was approximated by the adsorption and desorption of protein where the binding constant is the strong function of pH. Incorporating the buffering action model, accurate estimates of the elution profile of proton and proteins would be available. In present, we

are in progress with a differential model for computer calculations to simulate the column behavior incorporating the better buffering model [22].

CONCLUSIONS

Mixtures of four proteins, sperm whale myoglobin, horse myoglobin, transferrin and albumin with isoelectric points, 8.3, 6.8-7.8, 5.9, 4.7 respectively were successfully separated by chromatofocusing in a column packed with PBE 94 under pH gradients generated by the elution of Polybuffer.

In developing the pH gradient by an internal method, PBE 94 was superior to DEAE Bio-Gel A, and the shape of pH gradient depended on the concentration of equilibration buffer, and elution buffer.

Mixtures of four proteins were separated out in the order of PI values with high recovery yields, and the deviations of the elution pH from their PI's were less than 0.25 pH unit.

The internal elution pattern of myoglobin I in the gradient column was visually determined indicating the focusing effect of protein by pH.

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