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Isoelectric focusing field-flow fractionation

IV. Investigations on protein separations in the trapezoidal cross-section channel

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ABSTRACT

The first separation of three proteins (horse spleen ferritin, equine myoglobin and horse heart cytochrome *c*) by isoelectric focusing field-flow fractionation in a trapezoidal cross-section channel of 0.875 ml volume and 25 cm length is reported. Separation and elution are shown to proceed within about 1 h at a power application of about 1 W. The separation of the three proteins is demonstrated to be dependent on applied electric power, carrier ampholyte concentration and the concentrations of anolyte and catholyte. It follows from these data that the resolution is improved with increasing carrier ampholyte concentration and/or decreasing concentrations of the electrode solutions. The experimentally observed effects are in agreement with predictions made by computer simulation.

INTRODUCTION

Isoelectric focusing (IEF), particularly implemented in gels, has become an important method for the high-resolution separation and analysis of amphoteric compounds [1]. Recently, research was focused on free fluid IEF methods in order to extend the use of IEF to particles and cells which cannot pass through gels, for preparative IEF purposes in continuous-flow and recyling systems and to protein analysis in flowing streams which permit the use of detectors designed for liquid chromatography or capillary electrophoresis [2–4]. Separation in IEF is carried out in a pH gradient which is established between the anode and cathode. Amphoteric compounds migrate under the influence of the electric field until they concentrate at their isoelectric positions where the net charge (and therefore the migration) is zero. IEF is an equilibrium technique with a dynamic equilibrium between diffusion and other dispersing factors and the electrical focusing forces.

In addition to the electric field and pH gradient used in IEF, isoelectric focusing field-flow fractionation (IEF_4) employs the flow of the liquid carrier through a thin separation channel as a third factor affecting separation. The flow is perpendicular to the electric field and the flow velocity profile is determined by the geometry of the separation channel [5]. Amphoteric solutes are transported via isoelectric focusing to the equilibrium positions, where these compounds possess no net overall charge, and narrow focused solute zones with nearly Gaussian concentration distributions are formed. Provided that solutes exhibit different isoelectric points, they focus in different positions across the separation

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channel. Unequal flow velocities cause differential migration of focused solutes along the channel, *i.e.*, their longitudinal separation. Owing to the dimensions of the channels high electric field strengths can be applied with small voltages, thus keeping Joule heating at a low level. IEF_4 is an elution technique, its instrumental set-up being similar to that of high-performance liquid chromatography (HPLC) [5,6].

IEF_4 was experimentally introduced by Chmelík *et al.* [6] in the trapezoidal cross-section channel and by Thormann *et al.* [7] in the rectangular cross-section channel. The latter group named this technique electrical hyperlayer field flow fractionation following the terminology of Giddings [8]. So far, the formation of the pH gradient in a thin channel [9] and IEF_4 of a low-molecular-mass [10] and a high-molecular-mass compound [11] in the trapezoidal cross-section channel have been carefully studied. It was found that the pH gradient formation was sufficiently fast and reproducible for IEF_4. This work was devoted to the separation of three proteins under different experimental conditions and by computer simulation.

EXPERIMENTAL

Chemicals

All chemicals were of analytical-reagent grade. Cytochrome *c* from horse heart (CYTC, molecular mass M_r = 12 384, pI = 9.3) was obtained from Sigma (St. Louis, MO, USA) and ferretin from horse spleen (FER, M_r = 450 000, pI = 4.2–4.5) and equine myoglobin from skeletal muscle (MYO, M_r = 17 800, pI = 6.8–7.0) from Serva (Heidelberg, Germany). Ampholine (pH 3.5–10) was obtained from Pharmacia–LKB (Bromma, Sweden).

Instrumentation and experimental conditions

The scheme of the experimental IEF_4 arrangement has been described in detail elsewhere [6,7] and the experimental conditions used were selected on the basis of previous measurements [10,11]. The length of the trapezoidal cross-section channel was 25 cm, the height was 0.5 cm and the lengths of the two opposite walls of the trapezoid were 0.45 and 0.95 mm (volume 0.875 ml). PLGC ultrafiltration membranes (Millipore, Bedford, MA, USA) separated the focusing channel from the electrode compartments. Proteins were dissolved in carrier ampholyte solution and introduced with a four-port valve (featuring a 5-μl sample loop) through a capillary inlet situated 2 cm downstream from the carrier ampholyte inlet. Sample injection occurred over a period of 4 min using a Model 355 syringe pump (Sage Instruments, Cambridge, MA, USA). A Model 2150 HPLC pump (LKB, Bromma, Sweden) was employed to pump the carrier ampholyte solution at a pump rate of 10 μl/min during sampling and the subsequent 10-min relaxation period. The flow-rate was increased to 40 μl/min during elution. Eluting zones were monitored with a Model 2158 Uvicord SD photometric detector (LKB) at 405 nm and a Model 2210 Recorder (LKB). A Model 2297 Macrodrive 5 power supply (LKB) was used to apply up to 10 V (maximum current 100 mA). The electric field was applied during the entire experiment, including sample injection. A two-channel peristaltic pump (Vario Perpex, H. J. Guldener, Zürich, Switzerland) was used to pump solutions of acetic acid and sodium hydroxide through the anodic and cathodic electrode chambers respectively (pump rate 250 μl/min each). The carrier ampholyte and sample solutions were degassed by vacuum and filtered through 0.2-μm Nalgene (25-mm diameter) disposable syringe filters (Nalge, Rochester, NY).

Computer simulation

As described elsewhere [11], the PC-adapted software package of the transient electrophoretic model developed by Mosher *et al.* [12] was employed to predict the IEF behaviour of the proteins. This model is one-dimensional and isothermal and assumes the absence of fluid flows, hence it does not describe separation in IEF_4 but focusing at zero or very low flow-rates without elution. Ten biprotic carrier ampholytes were used to establish a pH gradient between acetic acid and sodium hydroxide. The pI values uniformly span the range 3–12 (ΔpI = 1). For each ampholyte, ΔpK was 2 and the ionic mobility was $3 \cdot 10^{-8}$ m^2/V · s. The pK and mobility value for acetic acid were 4.76 · 10^{-8} and 4.12 · 10^{-8} m^2/V · s, respectively, and the mobility of the sodium ion was $5.19 \cdot 10^{-8}$ m^2/V · s. The diffusion coefficients for CYTC and MYO were taken as $1.33 \cdot 10^{-6}$ [13] and $1.027 \cdot 10^{-6}$ cm^2/s [14], respectively. The diffusion coefficient for FER was taken to be equal to that of CYTC. The net charge *vs.* pH tables

employed are summarized in Table I. All simulations were performed with 67 grid points/cm and with a constant current density of 10 A/m^2. The three proteins were sampled as a Guassian peak (0.5 mM peak concentration) in the centre of the focusing column. The lengths of focusing space and electrode compartments were 1 and 0.25 cm, respectively [11].

TABLE I

pH DEPENDENCE OF THE IONIZATION OF PROTEINS

Ionization data were adopted with consideration of the charge of the iron ion from refs. 15 and 16 for CYTC and MYO, respectively. The data used for FER are hypothetical.

pH	Net charge		
	CYTC	MYO	FER
2.0	26.9		25
2.4			20
2.5	24.6		
3.0	21.3	24	
3.1			10
3.4			6.0
3.5	17.4	20	
3.9			3.0
4.0	14.7	16	
4.4			0
4.5	12.7	12	
4.8			−3.0
5.0	11.6	8.0	
5.4			−10
5.5	10.5	6.6	
6.0	9.9	4.6	
6.4		2.4	
6.5	9.7		
6.8		0	
7.0	9.4		
7.1			−14
7.2		−2.4	
7.5	9.1		
7.8		−4.6	
8.0	8.8		
8.5	8.6	−6.0	
8.9			−18
9.0	7.7	−8.0	−22
9.1			−26
9.2			−34
9.5	6.1	−12	
10.0	3.3	−16	
10.5	−0.1		
11.0	−5.3		

RESULTS AND DISCUSSION

The experimental conditions for the successful performance of an IEF$_4$ experiment were reported elsewhere [11]. It was found that (i) the sample has to be injected under applied electric power into the centre of a slowly flowing stream (10 μl/min), (ii) the relaxation time, *i.e.*, the time period necessary for formation of a focused zone, should be of the order of 10 min with no or minimum flow only and (iii) the efficiency decreases with increasing flow-rate of the carrier ampholyte solution.

For the characterization of individual compounds, the three proteins were first studied separately. The fractograms of FER, MYO and CYCT obtained at different applied voltages are depicted in Fig. 1. The IEF$_4$ procedure consists of three phases, sample injection, relaxation and elution. Typically each phase is executed at a different carrier flow-rate. Therefore, in order to be able to compare the influences of different experimental conditions on retention, all fractograms are expressed in units of elution volume (V_E) and not time. For all three proteins, there is a clear difference between the elution volume of unretained (bottom graphs) and retained protein (centre and top graphs). It was further found that with application of power FER has the lowest and CYTC the highest elution volume, with MYO eluting at volumes between those for the other two proteins. This observation agrees well with theory because with the configuration employed CYTC is expected to focus in the narrower part of the channel where elution is slow, MYO somewhere in the centre and FER towards the wider part where elution is fast (Fig. 2). The highest power level applied was 1 W, a value which was previously found to be safe for proper operation [11].

Fractograms depicting the separation of FER, MYO and CYTC at different power levels are presented in Fig. 3. Not surprisingly, there is no separation without application of the electric force field, as can be seen in the bottom graph. Application of a constant 5 V shows partial separation (centre graph) and almost complete resolution is obtained with 10 V (top graph). The last run was executed at a power level of about 1 W. This represents the first IEF$_4$ experiment showing the separation of three proteins and hence demonstrates the feasibility of this separation technique.

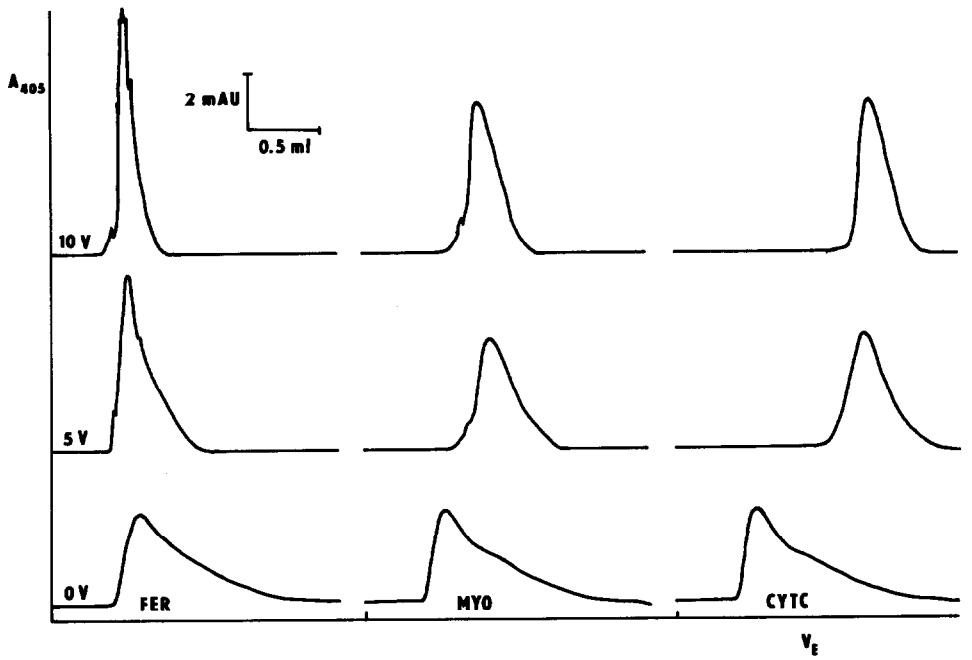

Fig. 1. Comparison of fractograms of FER (left hand panel), MYO (centre panel) and CYTC (right-hand panel) obtained without applied power (bottom graphs), with a constant 5 V (centre graphs) and a constant 10 V (top graphs). A 5-μl volume of sample containing either FER (3 μM), MYO (50 μM) or CYTC (50 μM) was injected in each instance. Carrier flow-rates under sample injection (4 min), relaxation (10 min) and elution were 10, 10 and 40 μl/min, respectively. The carrier ampholyte concentration was 2% (w/v) and the concentrations of anolyte and catholyte in the electrode compartments were 100 mM. The maximum current was 100 mA. The fractograms were monitored at 405 nm and are expressed as function of the elution volume, V_E. Origin of each fractogram indicated on V_E axis.

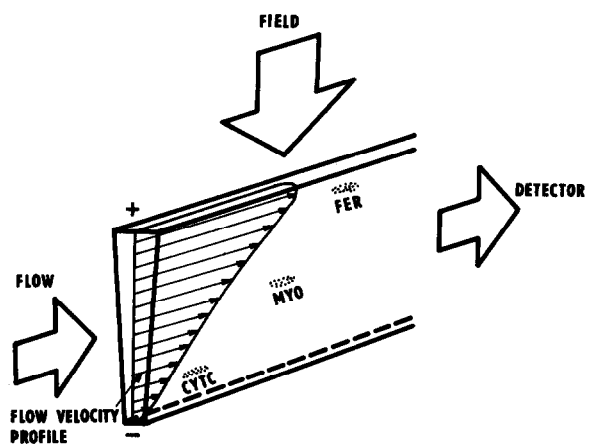

Fig. 2. Schematic representation of IEF_4 of CYTC, MYO and FER in the trapezoidal cross-section channel with the cathode on the narrower and the anode on the wider part of the channel.

The influence of the carrier ampholyte concentration on the separation of the three model proteins is depicted in Fig. 4. It is seen that the resolution of proteins is incomplete at a low (0.5%) Ampholine concentration (a) and significantly improved with 2% (c) compared with 1% (b) or 0.5% (a) Ampholine.

The influence of the buffer concentrations in the electrode chambers on resolution is presented in Fig. 5. Comparison of the fractograms obtained with (a) 100 and (b) 50 mM concentrations of anolyte and catholyte reveals that the proteins are better separated at the lower concentrations of the electrode solutions. The Ampholine concentration in that case was 2%. These findings are in good agreement with previous results which demonstrate that the pH gradient becomes shallower with either

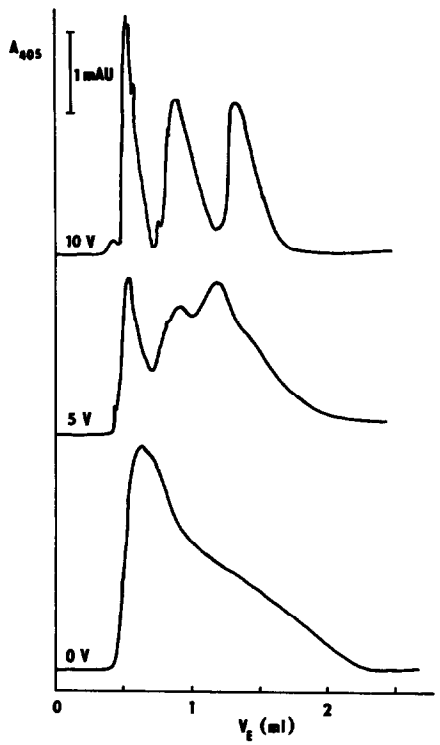

Fig. 3. Comparison of fractograms of a three-component protein mixture with FER (1 μM), MYO (17 μM) and CYTC (17 μM) at 0, 5 and 10 V. Other conditions as in Fig. 1.

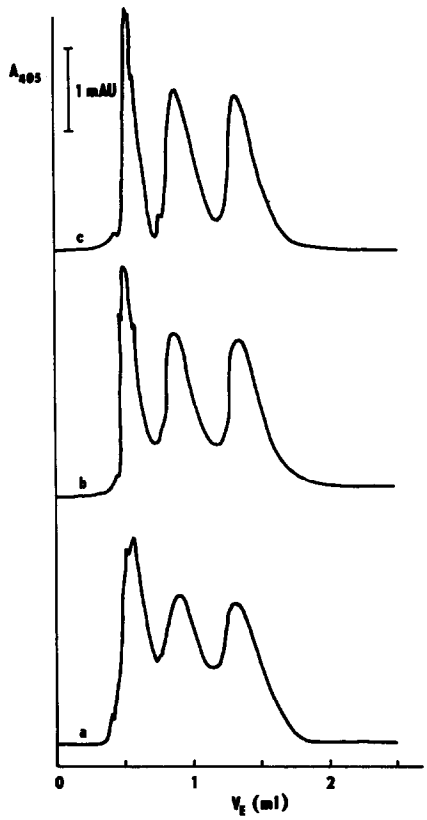

Fig. 4. Influence of carrier ampholyte concentration on protein separation with (a) 0.5, (b) 1 and (c) 2% (w/v) Ampholine. The applied voltage was 10 V. The sample solution was the same as in Fig. 3 and other conditions were as in Fig. 1.

an increasing concentration of carrier ampholytes and/or a decreasing concentration of electrolytes in the electrode chambers [9]. Generally, of course, protein separation is favoured in shallower pH gradients, which is exactly what is seen in the IEF_4 experiments.

Computer simulation was employed to confirm these basic dependences on protein separations. The computer-predicted dynamics of the three proteins with 2 mM concentrations of ampholytes are depicted in Fig. 6. The data in A and B were obtained with 100 and 50 mM electrode solutions, respectively. Note that the concentration profiles of ampholytes, catholyte and anolyte are not depicted, because they are very similar to those presented in ref. 11. With pulse sampling in the centre of the column, rapid protein separation is predicted (within 5 min) followed by focusing of the three proteins at characteristic locations. No significant change in the protein distributions is noted between 20 and 25 min of current flow, indicating that the separation phase was terminated between 15 and 20 min [17–19].

More interesting for the purposes of this paper are the pH gradients produced and the positions of the foci in relation to the initial ampholyte concentrations and the concentrations of the electrode solutions. The pH gradients after 25 min of current application for the two cases in Fig. 6 are shown in Fig. 7. Comparison of the two profiles reveals that a shallower gradient is predicted with 50 than with 100 mM electrode solutions and that the difference on the basic (cathodic) side is larger than that on the acidic side. This explains the fact that the protein foci are further apart in Fig. 6B compared with Fig. 6A, with the position of focused CYTC showing the largest effect on electrode solution concentration. Similar, but less pronounced, shifts are predicted with increasing carrier ampholyte concentrations

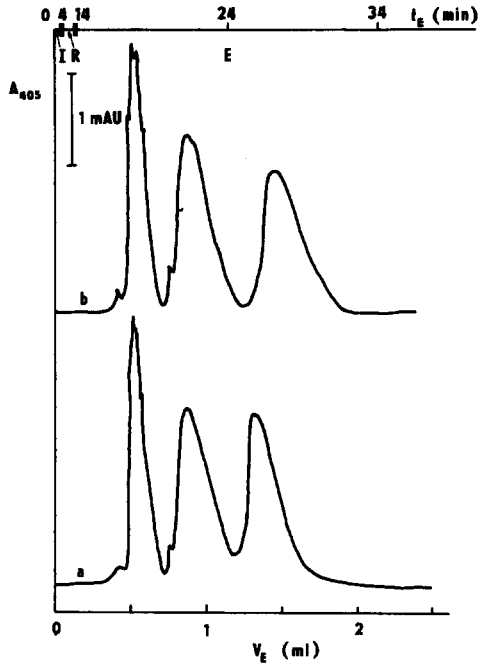

Fig. 5. Influence of anolyte and catholyte concentration on protein separation with (a) 100 and (b) 50 m*M* concentrations of acetic acid and sodium hydroxide, respectively. The applied voltage was a constant 10 V. The sample solution was the same as in Fig. 3 and other conditions were as in Fig. 1. The time axis with the three stages, sample injection (I), relaxation (R) and elution (E), is shown on the top.

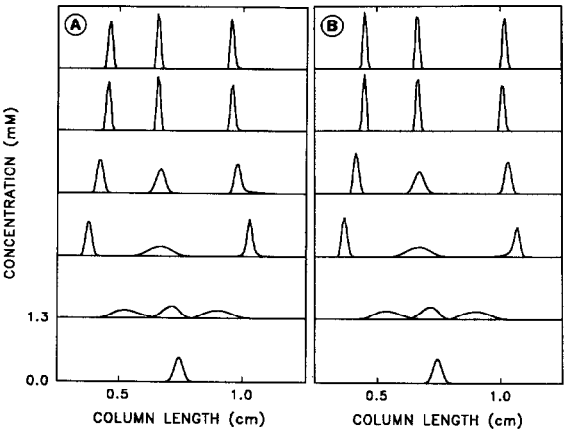

Fig. 6. Computer-simulated distribution of the three proteins after 0, 5, 10, 15, 20 and 25 min (from bottom to top) of current flow within the focusing space (from 0.25 to 1.25 cm column length) having (A) 100 and (B) 50 m*M* acetic acid and sodium hydroxide as anolyte and catholyte, respectively. The initial concentration of each carrier ampholyte was 2 m*M*. The anode is to the left. The distributions of the carrier ampholytes and the components from the electrode solutions are not shown. Each successive time point is offset from the previous one by a constant amount for presentation purposes. The left-hand, centre and right-hand foci are formed by FER, MYO and CYTC, respectively.

(data not shown). Hence the computer simulation data are in agreement with the experimental observations. These data demonstrate the applicability of the electrophoresis model without incorporation of fluids flow to predict basic focusing behaviour in IEF_4, and the establishment of proper flow conditions in the employed trapezoidal cross-section IEF_4 channel.

CONCLUSIONS

The data presented on the separation of proteins demonstrate the validity of the IEF_4 separation principle in a trapezoidal cross-section channel. The time period of an IEF_4 experiment in the channel employed is about 1 h and the applied electric power required is about 1 W. Separation is shown to be dependent on applied electric power, concentration of carrier ampholytes and concentration of electrode solutions. These effects are in agreement with predictions made by computer simulation. The feasibility of IEF_4 has been demonstrated, but not its resolving power or limit, because the investigated proteins are characterized by large differences in p*I* values. For a complete elucidation of the potential

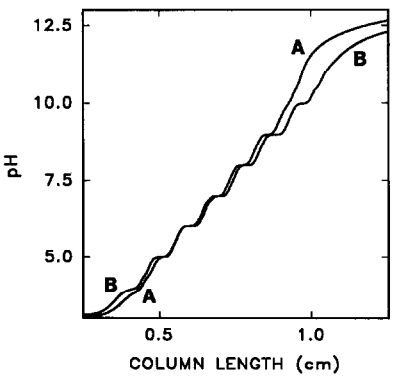

Fig. 7. Predicted pH gradients for the 25-min time points in Fig. 6 with electrode solution concentrations of (A) 100 and (B) 50 m*M*.

of IEF_4, further studies with other proteins and with channels of different lengths and cross-sectional areas will have to be executed. Further, comparative studies between IEF_4 and other IEF techniques in flowing streams are in progress and will be reported in due course.

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