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Isoelectric focusing field-flow fractionation and capillary isoelectric focusing with electroosmotic zone displacement

Two approaches to protein analysis in flowing streams

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ABSTRACT

A comparison of the separation of three proteins by two focusing methods in flowing streams, isoelectric focusing field-flow fractionation (IEF,) in a trapezoidal cross-section channel and capillary isoelectric focusing with electroosmotic zone displacement (cIEF) in an uncoated, open-tubular fused-silica capillary, is presented. In IEF, a hydrodynamic flow with a characteristic flow velocity profile as well as an electric force field and a chemical equilibrium (pH) gradient arranged perpendicular to the direction of flow are employed for separation. cIEF uses an electrokinetic plug flow with the separating electrical and chemical fields parallel to its direction. Protein zones are monitored by conventional detectors developed for liquid chromatography and capillary electrophoresis respectively. With current instruments, separation by cIEF is shown to be characterized by higher efficiency and resolution than separation in IEF_4. However, the latter method operates at much lower voltages and is simpler to apply for micropreparative purposes. The time intervals required for separation and analysis in the two methods are comparable.

INTRODUCTION

In isoelectric focusing (IEF), sample constituents are sorted in order of their isoelectric points in an equilibrium gradient. Proteins and other amphoteric compounds are separated in a pH gradient provided that their isoelectric points are different. Good resolution is favoured by both a low diffusion coefficient and a high mobility slope at the isoelectric point, conditions which are well satisfied by all proteins. A high electric field strength and a shallow pH gradient further enhance resolution. Depending on instrumental parameters, a resolving power of the order of 0.01 pH unit is typically achievable. Traditionally, IEF has been carried out in gels, requiring tedious, time-consuming preparation and protein staining procedures [1]. Compared with HPLC, the common practice of gel IEF is slow, labour intensive, prone to relatively poor reproducibility, difficult to quantitate and not accessible to simple automation. Therefore, in the past few years considerable attention has been focused on protein isoelectric focusing in capillaries [2–26].

First, free fluid focusing with the electric field parallel to the column axis was studied in capillaries of rectangular cross-section [2–6], in tubular glass capillaries [7–9], in PTFE capillaries [5,10,11] or in coated, open-tubular fused-silica capillaries of very small inner diameter (I.D.) [12–16]. These approaches operated with minimized electroosmosis

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in which stationary steady-state zone patterns were established. Zone detection occurred either by the use of array detection [2,3,5,6] or by UV absorption measurement towards the column end which required that after focusing the proteins had to be mobilized and swept past a stationary detector [7–16]. Essentially two approaches for mobilization were studied. First electrophoretic mobilization was achieved through power interruption after focusing and replacement of one of the two electrode buffers prior to reapplication of current [7–15]. The second method consisted in the use of hydrodynamic flow which was applied after focusing was attained and without interruption of the current flow [7,16].

In a second series of studies it was discovered that small amounts of a neutral polymer [hydroxypropylmethylcellulose (HPMC) or methylcellulose] added to the buffer allowed IEF analyses of proteins to be performed in untreated, open-tubular fused-silica capillaries, *i.e.*, in the presence of an electroosmotic flow along the separation axis [17–19]. In this approach the electroosmotic flow displaced the developing zone pattern towards and across the point of detection and made mobilization after focusing unnecessary. The added polymer provided a dynamic coating of the capillary surface which reduced both the protein-wall interactions and the electroosmotic flow. This, in addition to the plug flow characteristics of electroosmosis, were important prerequisites for low sample dispersion and therefore for efficient focusing.

In a third approach, focusing was investigated in a flowing stream with the electric force field being perpendicular to the column axis and flow. This method, termed isoelectric focusing field-flow fractionation (IEF_4), was experimentally introduced by Chmelik et al. [20] in a trapezoidal cross-section channel and by Thormann *et al.* [21] in a rectangular cross-section channel. The latter group named this technique electrical hyperlayer field-flow factionation, following the terminology of Giddings [22]. So far, the formation of the pH gradient in a thin channel [23], IEF_4 of a low-molecular-mass substance [24] and a high-molecular-mass compound [25] and the separation of three proteins [26] in a trapezoidal cross-section channel have been carefully studied.

This paper is devoted to the elucidation of the differences, similarities, advantages and disadvantages of the two focusing methods in flowing streams, *viz.*, IEF_4 and capillary isoelectric focusing with electroosmotic zone displacement (cIEF).

EXPERIMENTAL

Chemicals

All chemicals were of analytical-reagent grade. Cytochrome c from horse heart (CYTC; M_r 12 384, pI 9.3) and HPMC were obtained from Sigma (St. Louis, MO, USA), ferritin from horse spleen (FER; M_r 450 000, pI 4.24.5) and equine myoglobin from skeletal muscle (MYO; M_r 17 800, pI 6.8–7.0) from Serva (Heidelberg, Germany) and Ampholine (pH 3.5510) from Pharmacia-LKB (Bromma, Sweden).

Instrumentation and experimental conditions

For IEF_4 the experimental set-up was described in detail elsewhere and the experimental conditions used were selected on the basis of previous measurements [25,26]. 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 2% (w/v) carrier ampholyte solution and introduced with a four-port valve (featuring a 5-μl sample loop) through a capillary inlet placed 2 cm downstream from the carrier ampholyte inlet. The concentrations of CYTC, MYO and FER were 17, 17 and 1 μM, respectively. Sample injection occurred over a period of 4 min using a Model 355 syringe pump (Sage Instruments, Cambridge, MA, USA). A Model 2 150 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 IO-min relaxation period. The flow-rate was increased to 40 μl/min during elution. Eluting zones were monitored with a Model 2158 Uvicord SD (LKB, Bromma, Sweden) photometric detector 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 Vario Perpex two-channel peristaltic pump (H. J.

Guldener, Zurich, Switzerland) was used to pump solutions of acetic acid and sodium hydroxide (50 m*M* each) 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, USA).

A laboratory-made instrument was employed for cIEF [19]. It featured a 75 μm I.D. fused-silica capillary of about 90 cm length (Polymicro Technologies, Phoenix, AZ, USA) together with a UVIS 206 PHD fast-scanning multi-wavelength detector and a No. 9550-0155 capillary detector cell (both from Linear Instruments, Reno, NV, USA). The effective separation distance was about 70 cm between the anodic capillary end and the detection window. No cooling of the capillary was provided. Two 50-ml plastic bottles served as electrode vessels, containing 10 m*M* phosphoric acid (anolyte) and 20 m*M* NaOH with 0.1% HPMC (catholyte). Current was applied at a constant voltage (20 kV) with a Model HCN 14-20000 power supply (FUG Elektronik, Rosenheim, Germany). A VacTorr 150 vacuum pump (CGA/Precision Scientific, Chicago, IL, USA) was used to rinse the capillary. New capillaries were first rinsed with 1 *M* NaOH (20 min) and then 0.1 A4 NaOH containing 0.3% HPMC (10 min). The latter solution was also used to condition the capillary at the beginning of a series of experiments (10-min wash). Before each run the capillary was cleaned with catholyte for at least 10 min. Sample proteins were dissolved in 2.5% Ampholine solution without the addition of HMPC. The concentrations of CYTC, MYO and FER were 4.8, 3.4 and 0.1 μ*M*, respectively. Sample application occurred manually via gravity through lifting the capillary end (dipped into the sample vial) to a height of 65 cm for 6 min.

RESULTS AND DISCUSSION

Before turning to the comparison of the experimental results, basic differences and similarities of the methods of interest and the performance of the experiments are considered. IEF_4 and cIEF differ fundamentally in two respects, the orientation of the electric field with respect to column axis and flow, and the shape of the velocity profile. In IEF_4 the force field is applied perpendicularly to the column axis and hydrodynamic flow with a characteristic flow profile being employed for elution. The shape of the flow velocity profile, which is determined by the geometry of the separation channel [27], is a prerequisite for sequential elution of the separated compounds (Figs. 1A and 2). In cIEF the electric field is parallel to the column axis and an electroosmotic flow with a plug profile is utilized solely for the displacement of the entire zone structure towards and across the detector (Fig. 1A).

In addition to the electric field and pH gradient used in IEF, IEF_4 employs the flow of the liquid carrier through a thin separation channel as a third factor affecting separation. 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 channel (Fig. 2B). Unequal flow velocities cause differential migration of focused solutes along the channel, *i.e.*, their longitudinal separation (Fig. 2C). Owing to the dimensions of the channel a high electric field strength can be

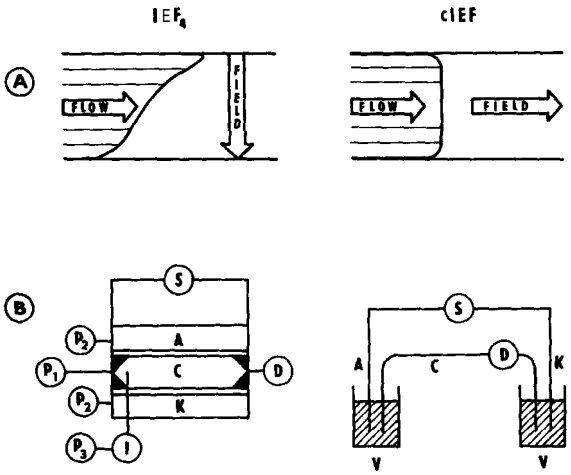

Fig. 1. IEF_4 in the trapezoidal cross-section channel and IEF with electroosmotic zone displacement with (A) orientation of electric field and flow, as well as the characteristic flow velocity profiles, and (B) schematic representations of the experimental set-ups. A = Anode; K = cathode; C = channel or capillary; D = detector; I = injection port; P_1 = carrier ampholyte pump; P_2 = electrolyte pumps; P_3 = sample pump; S = power supply; V = electrolyte vessel.

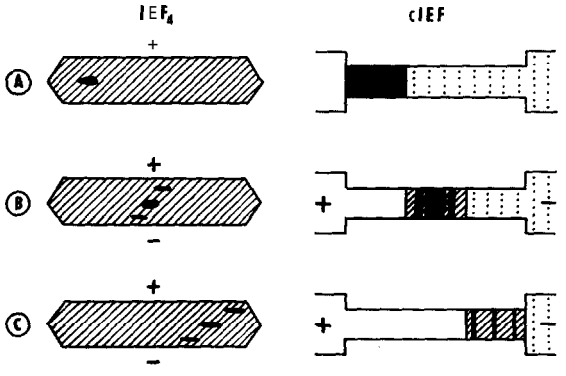

Fig. 2. Separation in IEF_4 and cIEF with (A) sample application, (B) focusing and (C) elution. Proteins are represented by the black areas, carrier ampholytes by the hatched areas and catholyte and anolyte in cIEF by the dotted and white areas, respectively. The directions of both hydrodynamic (IEF,) and electroosmotic (cIEF) flows are from left to right.

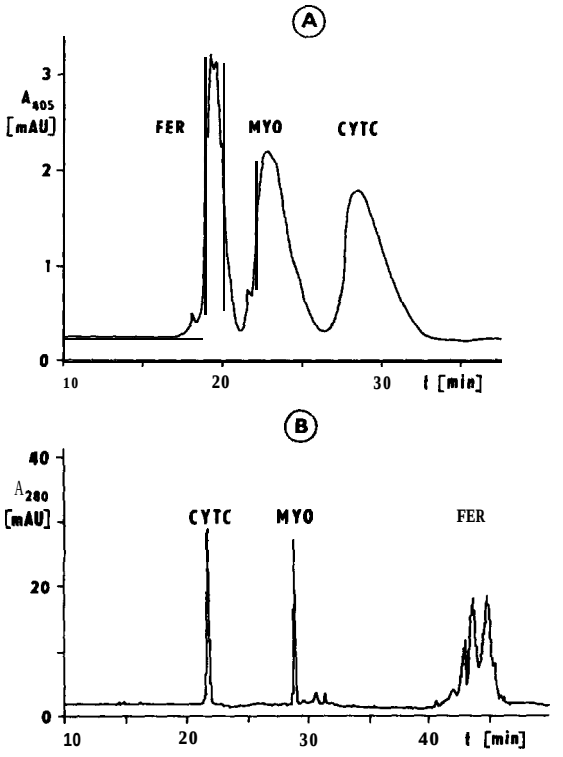

Fig. 3. Separation of CYTC, MYO and FER by (A) IEF_4 in a trapezoidal cross-section channel of 25 cm length (volume 0.875 ml) and (B) cIEF using a fused-silica capillary of 75 μm I.D. and 90 cm total length (volume 4 μl). The detection wavelength (detector positions) were 405 nm (off-column) and 280 nm (on-column), respectively. The power levels applied were about 1 and 0.1 W, respectively, values which were previously found to guarantee safe operation (refs. 26 and 19. respectively). For experimental details, see text.

applied with a fairly small voltage, this keeping Joule heating at a low level. IEF_4 is an elution technique, its instrumental set-up being similar to that of HPLC [20,21]. The IEF_4 procedure consists of three phases, sample injection, relaxation and elution. Typically each phase is executed at a different carrier flow-rate. The experimental conditions for the successful performance of an IEF_4 experiment in the trapezoidal cross-section channel of 0.875-ml volume have been reported in other papers [25,26]. 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 minimal flow only and (iii) the efficiency decreases with increasing flow-rate of the carrier ampholyte solution. A fractogram depicting the separation of FER, MYO and CYTC is presented in Fig. 3A.

cIEF is performed in an uncoated, open-tubular fused-silica capillary of typically 75 μm I.D. The experimental arrangement used in this work is depicted schematically in Figs. 1 B and 2. An experiment proceeds as follows. First the entire capillary is filled with the catholyte containing a neutral polymer. Sample composed of carrier ampholytes and proteins is introduced at the anodic capillary end and is occupying 530% of the effective capillary length (Fig. 2A). After power application two electrokinetic effects occur simultaneously (Fig. 2B), the formation of a longitudinal pH gradient and the separation of proteins (isoelectric focusing), and, owing to the negative surface charge of untreated fused silica, the displacement of the entire pattern towards the cathode (electroosmosis). Basic proteins, such as CYTC, reach the detector prior to neutral and acidic proteins, as is seen with the example shown in Fig. 3B. This experiment, performed in a capillary of 4-μl volume, was executed with a lower (about 18-, 25- and 50-fold for CYTC, MYO and FER, respectively) amount of protein compared to that employed in IEF_4 (Fig. 3A). The protein load per unit of column volume, however, was higher in the cIEF experiment.

From the experimental results for the two methods (Fig. 3), it is apparent that the three proteins are eluted in reverse order. In IEF_4, FER has the lowest and CYTC the highest elution time, and MYO elutes between them. This observation agrees well with theory because with the configuration emloyed (anode at the wider side of the channel) 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. In cIEF the order of elution is determined by the electroosmotic flow, which is in the direction of the cathode. Therefore, the compound with the highest isoelectric point reaches the detector first and the elution order is according to decreasing isoelectric points. Hence, in the experiments presented, the sequence in cIEF has to be the opposite to that in IEF_4. It is important to add that reversal of the polarity in IEF_4 together with an exchange of the electrode buffers would simply reverse the elution order of the proteins in that method. This, however, does not apply to cIEF.

Comparison of the traces in Fig. 3 further reveals that both the efficiency (peak width) and resolution are much higher in cIEF. Separation of the three proteins is essentially achieved in both approaches. By p*I* discrimination as employed in IEF_4 and cIEF, CYTC is a single-component protein. The fractogram and electropherogram are both characterized by a single peak. However, differenecs are observed with MYO and FER, proteins which contain several components with different p*I* values. MYO contains minor compounds which are more acidic than the main protein. These are well resolved in cIEF and detected as a significant shoulder or unresolved peak in IEF_4. FER is a more complex protein containing many isoforms of different p*I*, up to six being detected by cIEF, whereas the fractogram showing several shoulders only.

In both methods, the resolution is dependent on the slope of the pH gradient and the power applied. Increased resolution is obtained by making the equilibrium gradient shallower and/or the electric field larger. In IEF_4 different experimental parameters, including the time interval of sample loading and the choice and concentration of the electrolytes in the electrode compartments, also have an influence on resolution. For example, with 10-min sample loading, the resolution for FER was higher than that obtained with a 4-min time interval shown in Fig. 3A (data not shown). The efficiency in IEF_4 is dependent on the relaxation process and the elution flow-rate, whereas in cIEF the efficiency is strongly influenced by the temporal behaviour of the electroosmotic flow.

From a theoretical point of view, IEF_4 in a trapezoidal cross-section channel is not as efficient as IEF_4 in a rectangular cross-section channel of high aspect ratio [28]. However, results obtained so far in the latter configuration have not confirmed the theoretical expectations [21]. Technical and material problems encountered in the construction of the rectangular cross-section channel are believed to be the reason for this. It appears to be very difficult to place narrowly (a few tenths of 1 mm) two flat membranes which define the ribbon-like channel. Further, establishment of the lateral pH gradient is hampered by the non-ideal behaviour of the membranes under current flow. Further efforts will have to be devoted to exploit new materials for the construction of a more efficient channel of rectangular cross-section. cIEF as described here and elsewhere [19] is different to the cIEF approach reported by Mazzeo and Krull [17,181. They used a configuration in which the column is initially completely filled with the sample, requiring the further addition of a strong base to the sample in order to be able to detect basic proteins [10,15]. Because of the relatively short initial sample zone in our fully dynamic approach, this disadvantage is not encountered. It should be added that the dynamics of cIEF in the presence of electroosmotic zone displacement are not yet fully understood. Further investigations using both experimental studies and theoretical descriptions of the underlying processes will provide further insight into this methodology and will lead to the complete elucidation of the analytical capabilities of cIEF.

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