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AUTOMATED RECYCLING FREE FLUID ISOTACHOPHORESIS

PRINCIPLE, INSTRUMENTATION AND FIRST RESULTS

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SUMMARY

Most investigations involving preparative isotachophoresis report the use of solid support media in which sample loads of up to 1 g are fractionated within several hours. Larger throughputs and simpler recovery of purified proteins have been achieved by continuous flow isotachophoresis. This method is based on a thin film of fluid flowing between two parallel plates with the electric field applied perpendicular to flow direction. Both the leading and the terminating electrolytes, as well as the sample, are continuously admitted to one end of the electrophoretic chamber and are collected through an array of outlet tubes at the other. In recycling isotachophoresis the effluent from each channel is reinjected into the cell through a corresponding influent port. The development of automated recycling isotachophoresis with a computer controlled counterflow of leading electrolyte is described together with its prospective use as a downstream processing unit operation in biotechnology.

INTRODUCTION

In isotachophoresis (ITP), separation is based upon differences in electrophoretic mobilities and carried out in a discontinuous background buffer system. The buffers are chosen so that there is a distinct mobility difference between the two buffers, the leading constituent having a higher electrophoretic mobility than the terminating one. The sample components of interest must have mobilities intermediate between those of the two electrolyte constituents in order to migrate isotachophoretically between the leader and terminator. The completion of a separation based upon ITP is characterized by a migrating steady state consisting of adjacent sample zones[1-8]. Complex protein samples are typically subfractionated by adding suitable

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spacer components (simple buffer constituents or synthetic ampholyte mixtures such as Ampholine[7,8]). In addition, a small amount of a dye is often included for the visualization of the leading edge of the ITP stack. The purification problems of the modern biotechnology industry have prompted in recent years a renewed interest in preparative electrophoresis[9]. Among the various electrophoretic methods, preparative ITP is the least explored. Nevertheless, it constitutes an attractive purification methodology because of (i) its high resolution coupled with high protein concentration, (ii) the achievement of high throughput, (iii) the control over the fractionation pH which is important in order to minimize protein precipitation and denaturation, problems which are specific for isoelectric focusing (IEF), (iv) the high efficiency of the process in terms of utilizing the power for separation, and (v) minimum consumption of inexpensive buffer solutions relative to the amount of protein processed[10–14]. Factors (iv) and (v) guarantee economical operation. Analytical ITP is complementary and essential to preparative scale ITP. It was used to predict the order of the individual components in the stack, whether a given pair of spacers will indeed bracket the protein in the stack and whether the choice of leading and terminating electrolytes is appropriate for the system under consideration[10,15]. The advantage of the analytical system is that the scale of the experiment is on the micro- to nanogram scale and that the analysis may be performed within a few minutes if a capillary-type instrument with on-line detection is used.

Most investigations involving preparative ITP report the use of solid support media. Vertical columns filled with polyacrylamide, agarose or granulated gels (Sephadex[10]), and procedures using horizontal gel slabs of about 3 mm thickness, are the favorite approaches[11]. Columns are equipped with an elution chamber at the end permitting transportation of the emerging sample components through a detector and into the fraction collector. Gel slabs are cut with an array of parallel blades, followed by elution of the sample constituents from each gel segment. Small scale preparative ITP in polyacrylamide gel filled glass tubes of 5 to 6 mm I.D. and procedures using annular gels wrapped arround a glass tube (hollow cylinder technique[14]), are alternative and less popular methods. When fractionating proteins in solid support media, sample loads of typically 1 g are applied yielding < 50 mg quantities of specific components in a matter of several hours. Further scale up is very difficult. Removal of the heat produced, the laborious set up of the column or slab, as well as the tedious recovery after separation, are the major obstacles.

Continuous flow electrophoretic methods[16–18], in particular continuous flow isotachophoresis (CFITP)[18–29], with the electric field applied perpendicular to the flow direction, appear to possess the capacity required for downstream processing of fermentation products in biotechnology (> 10 g protein/day). Both the leader and the terminator, as well as the sample, are continuously admitted to one end of the electrophoretic chamber and are fractionated at the other. Early versions utilized filter-paper, membrane compartmentation or density gradients for fluid stabilization[19,20]. More recent setups comprise a thin film of fluid flowing between two parallel plates[21–30]. An example of the latter type of instrument is the Elphor VaP 21 continuous flow electrophoresis apparatus of Bender and Hobein (Munich, F.R.G.) for which a maximum processing rate of 5 g protein per hour was reported in the CFITP mode[28]. CFITP represents an elegant method for the continuous concentration of dilute protein solutions. The positions of the sample zones at the exit are

dependent on the electric field strength, temperature, flow-rate, sample and buffer composition. In the case of small mobility differences, considerable separation distances are needed. To overcome this problem fractionation can be carried out under counter current conditions comprised of a continuous introduction of leading electrolyte on the leading side and withdrawal of an equal amount of fluid on the terminating side. By suitable adjustment of the electric field strength, the mixture being separated travels through the center of the cell with the leading boundary virtually immobilized. To obtain long term stability and proper control with this technique, appropriate automatic feedback devices would need to be incorporated as suggested many years ago by Fawcett[23]. The optical monitor at the bottom of the VaP 21 chamber, consisting of a scanning light beam together with a photosensor, comprises a first approach for on-line detection.

In another approach the principle of recycling electrophoresis, where the effluent from each channel is continuously reinjected into the cell through the corresponding influent port[9,15,31–35], was applied to IEF[9,31–33] and to zone electrophoresis[9,33], and was recently proposed for ITP[34,35]. Similarly, Ivory and co-workers[36–38] described a recycling zone electrophoresis approach where the effluent from each channel is not reinjected into the corresponding influent port but backshifted by a specified number of input channels. The development of automated ITP in a recycling mode (RITP) is the objective of the research endeavor reported here.

MATERIALS AND METHODS

A recycling free flow focusing apparatus (RF3) has recently been designed and constructed in our laboratory in which fluid flows through a narrow channel (0.75 mm) between two flat plates[9]. The fluid residence time in the apparatus is only a few seconds. This impacts a remarkable stability due to fluid dynamics[39]. On the other hand, the short residence time prevents complete separation in a single pass through the separation chamber thus recycling is essential to achieve steady state focusing. This apparatus has been modified for use in the RITP mode. A schematic representation of the entire RITP set up is shown in Fig. 1a and that of the separation chamber in Fig. 1b. ITP takes place within a thin flowing stream in a 35 cm long rectangular chamber having a width of 5.5 cm (volume 14.44 ml). Arrays of 48 inlet and outlet ports define the bottom and top respectively, and dialysis membranes isolate the separation chamber from the electrode compartments. The two channels at the extreme edges represent the fluid between the membrane and the electrophoretic chamber (Fig. 2). Fluid of 44 channels flows through the 5.5 cm wide chamber. The system developed (Fig. 1) consists of the separation chamber along with accessory components [recirculating pump, heat exchanger, flow stabilizing equipment and power supply (EC 600; EC Apparatus Corp., St. Petersburg, FL, U.S.A.)], a UV sensor (2138 Uvicord S; LKB, Bromma, Sweden) for determining the position of the interface between the leading electrolyte and the sample components (front), a method of sample injection, a method of applying a bulk counterflow of leading electrolyte (syringe pump No. 351; Sage Instruments, Orion, Cambridge, MA, U.S.A.) and a computer system for data gathering/data treatment/data storage and control [Commodore 64 (C-64)].

The separation chamber is the most complex portion of the device. It consists of

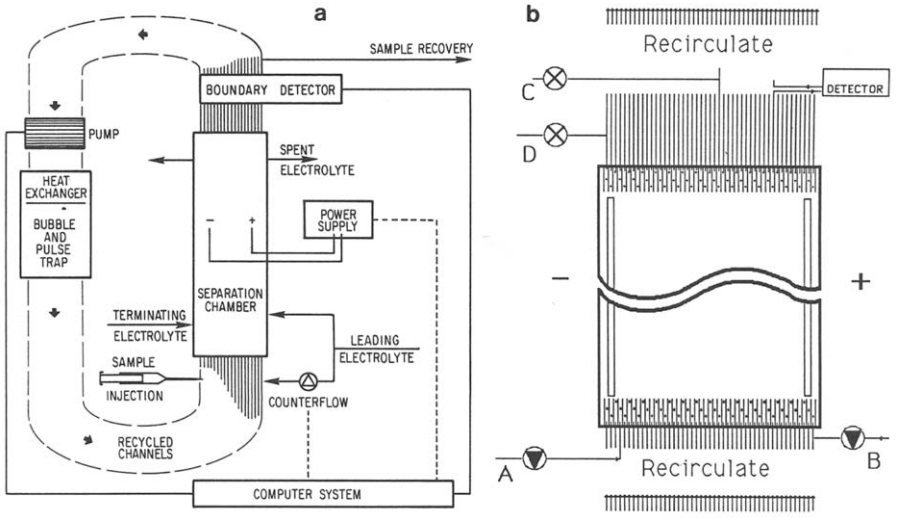

Fig. 1. Schematic representation of the RF3 configured for RITP (a) and the RITP separation chamber (b). A = Sample inlet; B = counterflow inlet; C = center drain valve; D = counterflow drain valve.

a securely braced piece of 3.8 cm plexiglass into which has been machined a cavity to a depth of 0.75 mm; at both ends of this cavity are 1.25 to 1.90 cm partitions (0.25 mm thick, 1.25 mm center to center distance) which serve the purpose of stabilizing the flow prior to entrance into the cell. Each channel formed by these partitions is connected to one tube from the manifold matrix. The electrodes are mounted in chambers on the front of the separation chamber and are connected to the flowing fluid via a thin slot (0.75 mm) running the length of the chamber (Fig. 2). The cell was attached

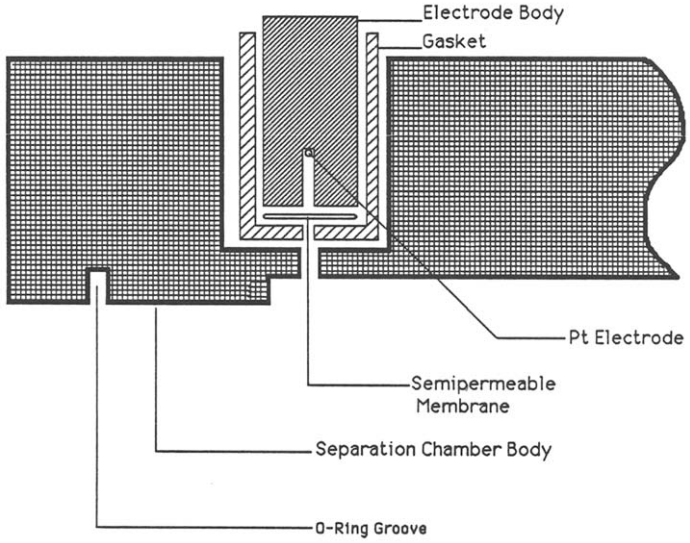

Fig. 2. Cut-away view of the separation chamber detailing the electrode mounting chambers.

to a thermostated cooling plate and sealed using a pair of O rings. Recirculation of the process fluid is provided by a 52-channel peristaltic pump accurate to within 10% per channel. The pump has an adjustable shoe so that the amount of pressure on the tubing may be carefully adjusted to provide minimum pulsing at the inlet and outlet. The flow-rate of process fluid through the pump may be above 500 ml/min (> 12 ml/min per channel). The third major portion of the system is the combined array of heat exchangers/bubble and pulse traps. Serving as the primary fluid reservoir of the system they provide for the most straightforward modification of the process volume. These reservoirs maintain the separation of all 48 fractions (about 4.5 ml each in the current model) of the process volume while providing uniform cooling and storage.

In its simplest implementation, RITP constitutes a batch process. A typical experiment proceeds as follows. The terminator is used as the electrode solution on the side opposite to the detector (Fig. 1b), whereas leading electrolyte with a two to ten-fold higher concentration than employed in the separation chamber is in the other electrode compartment. The electrophoresis chamber, the tubing loops and the channel reservoirs are filled with leading electrolyte (about 250 ml in the current model) which is recirculated until the fluid has thermally equilibrated. The sample is then injected into tube No. 4 (A) and the power (50 to 200 W; constant current of 50 to 100 mA) is applied. As soon as the front is detected (typically around channel 40), the center drain tube (C) is closed and a drain tube on the terminator side (channel 1, D) is opened. When the absorbance at the detector has reached some preset value, the syringe pump (B) is toggled on or off to control the position of the stack. The volume drained from the cell exits via channel 1 (D) and a net movement of fluid is established in a direction opposite to that of electromigration. Upon completion of the separation, the power is switched off and all pumps are stopped. A fraction collector is attached and 48 individual fractions are collected and analyzed separately for pH, conductivity, absorbance and specific activity. Selected fractions were also analyzed by capillary ITP using the Tachophor 2127 analyzer (LKB) featuring a 0.5 mm I.D. PTFE capillary and UV and conductivity detection at the column end.

Monitoring of the sample stack is based on flow through UV absorbance using the LKB 2138 Uvicord S. The C-64 was interfaced to the system employing a modified commercially available analog to digital/digital to analog (A/D, D/A) converter (MW-611; Microworld R&D, Lakewood, CO, U.S.A.)[35]. This board also has sixteen single ended discrete outputs (solid state darlington relays) capable of switching 30 V.d.c. at approximately 100 mA. A 12-V d.c. power supply was built into the interface box and a separate relay box was built to hold up to sixteen 12-V d.c. relays (DPDT) with contacts rated at 1000 V. These relays were chosen to withstand voltages associated with conductivity detection (not yet implemented). A relay was employed to toggle the syringe pump in response to the UV absorbance measurement. The interface between the Uvicord and the A/D converter required the construction of a 50-fold linear operational amplifier with overvoltage and reverse polarity protection. This amplifier converted the 0–100 mV Uvicord signal to the 0–5 V signal required by the A/D converter. The computer provides for measurements to be taken of the order of one per second and the logging of all data every fifteen clock seconds. Three computer programs were written for the data collection and manipulation. Fig. 3 displays an example of the three programs in operation. The dotted line in Fig. 3a is the set control absorbance maintained by the computer and the solid line is the actual

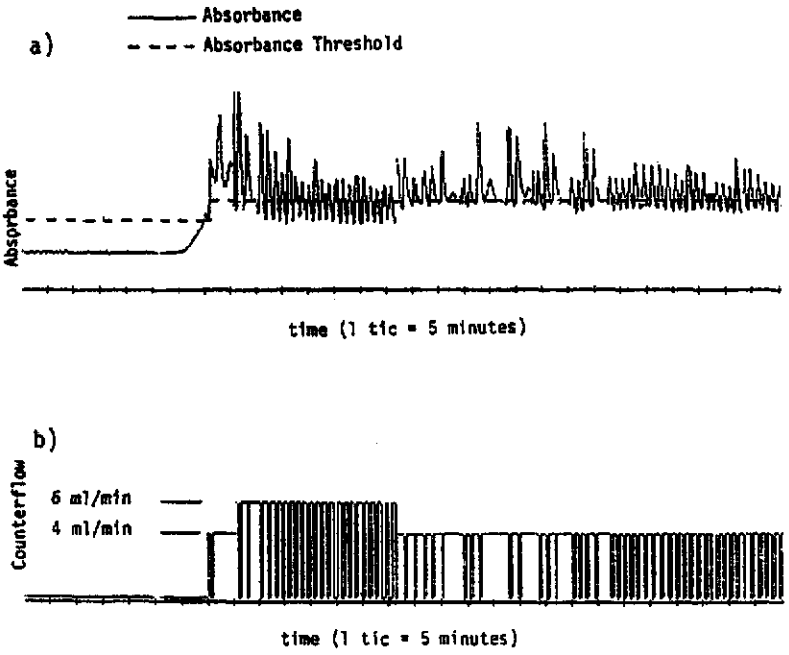

Fig. 3. Example of tracing of UV absorbance *vs.* time (a) and of counterflow *vs.* time (b) during RITP.

absorbance recorded by the computer. Fig. 3b is a record of the counterflow of leading electrolyte (4 to 6 ml/min) applied to the system by the syringe pump.

RESULTS AND DISCUSSION

Adjacent sample components may overlap in the steady state if the quantity is insufficient to form ITP plateaus[40]. Separation, however, can be achieved (i) by using spacer components[7,8] and (ii) by the employment of leading/terminating buffers which selectively stack the component of interest. Fig. 4a shows RITP data of two anionic dyes, amaranth red and fluorescein, with acetate as a spacing constituent. Amaranth red absorbs at 525 nm, fluorescein at 450 nm and the two sample components are detected at 254 nm. The data of Fig. 4a were gathered after 180 min of current flow (current density = 0.021 A/cm^2). Counterflow was applied for 94 min at a rate of about 3 ml/min. Selected fractions from these experiments were analyzed by capillary ITP using the Tachophor 2127 (Fig. 4b). These measurements confirmed the separation of the two dyes shown with the absorbance data of Fig. 4. Resolution in RITP in a 0.75-mm thin fluid film, however, is somewhat lower compared to that in the 0.5 mm I.D. capillary. Nevertheless, the system behaves in ITP manner in two respects, (i) the distinct step gradients in pH and conductivity and (ii) the relative spacing of the two dyes by addition of varying amounts of acetate (data not shown). Also, the zone structure could be immobilized by application of a pulsed counterflow (Fig. 3b).

An anionic protein separation is presented in Fig. 5. A 5 m*M* solution hydro-

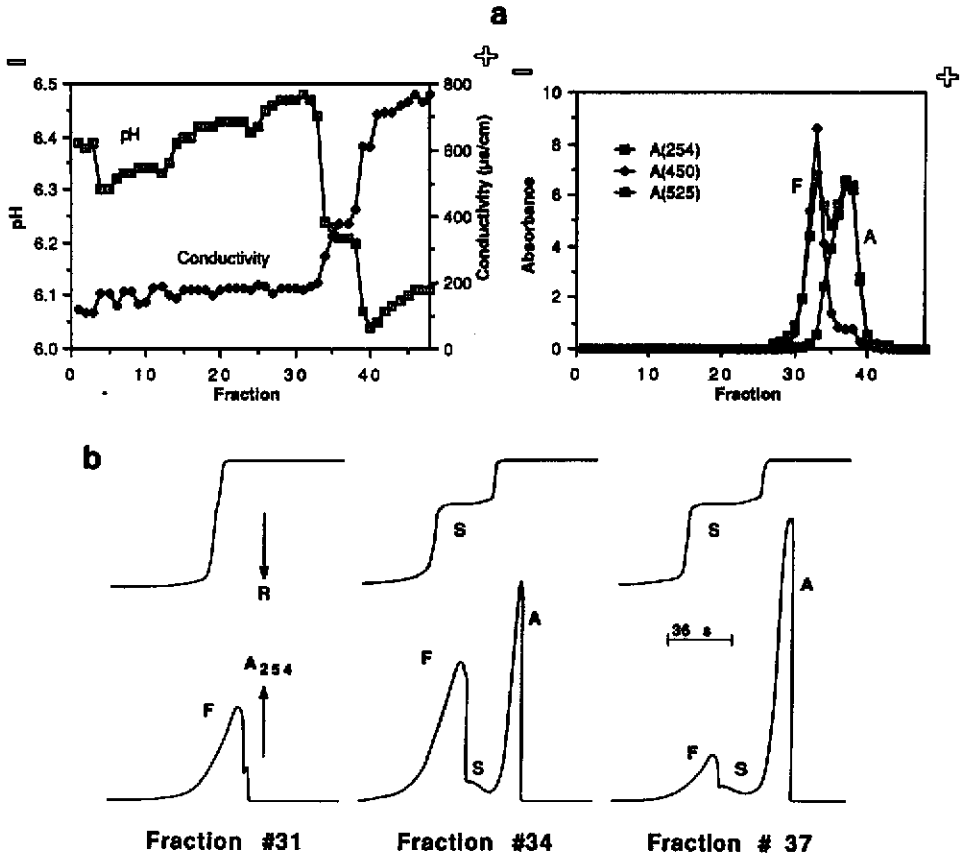

Fig. 4. (a) Anionic RITP of amaranth red (A), acetate (S) and fluorescein (F) using 10 m*M* hydrochloric acid-histidine (pH 6) as the leading electrolyte. A 20 m*M* solution of the same electrolyte was the anolyte. A 20 m*M* solution of 2-(4-morpholino)ethanesulfonic acid (MES) adjusted with histidine to pH 6 was employed as the catholyte. A constant current of 55 mA was applied (power level 60 W). A 2-ml sample containing 5 m*M* of each dye and 150 m*M* acetate was injected. (b) Anionic capillary ITP analyses of fractions 31, 34 and 37 of this RITP run using a 22-cm PTFE capillary of 0.5 mm I.D. The same electrolyte system was employed. A 5-μl volume of each fraction was injected and the experiments were performed at a constant 150 μA. The time of analysis was about 5 min. The measured conductivity is given as resistance (R).

chloric acid adjusted with ammediol to pH 9.5 was employed as a leader whereas ε-aminocaproic acid (EACA) was the terminating constituent. Bovine serum albumin was separated from canine hemoglobin by use of glycine as a spacer. Such low-molecular-weight spacers have the particular advantage of being easily removed from the final purified product. The two proteins were assayed spectrophotometrically at 415 nm (hemoglobin) and 602 nm (blue stained albumin). The data shown are corrected for the absorption of hemoglobin at 602 nm and blue stained albumin at 415 nm. The data shown in Fig. 6 illustrate the loading capacity of the RITP apparatus described. A 2.88-g amount of bovine albumin was processed employing 5 m*M* hydrochloric acid with ammediol (pH 9.17) as the leading electrolyte and EACA as the terminating constituent. The fractions were collected after 39 min of current flow at a

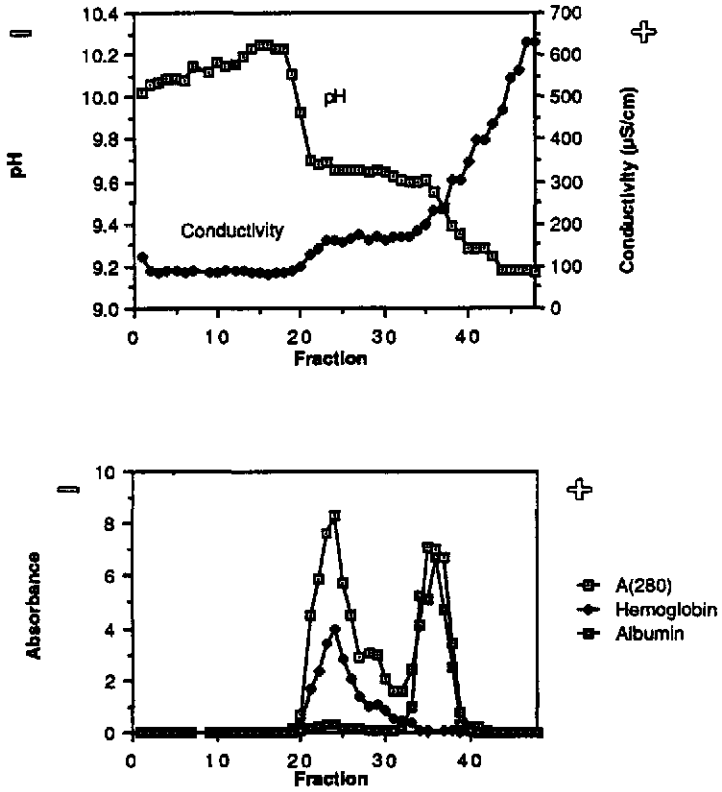

Fig. 5. Anionic RITP of 345 mg bovine albumin and 350 mg canine hemoglobin with 38 mg of glycine as a spacing constituent. A solution of 5 m*M* hydrochloric acid adjusted with ammediol to pH 9.54 was used as the leading electrolyte. A 60 m*M* solution of EACA with ammediol at pH 10.47 was the catholyte and 15 m*M* hydrochloric acid with ammediol was the anolyte. Profiles for total protein (280 nm), albumin and hemoglobin are shown.

current density of 0.038 A/cm^2 (counterflow for 9 min). An albumin recovery of 83% was obtained. The steady state protein concentration under these conditions was found to be about 8 mg/ml (0.8%, w/v), a value which compares well with computer simulation data and experimental values obtained with other electrophoretic instrumentation[41]. The total protein load for the RITP apparatus used is in excess of 3 g, the separation of which could be achieved in the order of 30 min leading to a protein processing rate of 6 g/h.

Cationic RITP data depicting the stacking of prepurified lectins from lentils (lens culinaris) are shown in Fig. 7. A 2.5-ml volume of a lentil extract which was prepurified with the Biostream separator in a recycling mode[42] was injected into 10 m*M* potassium acetate, processed at a constant current density of 0.038 A/cm^2 , and held around channel 39 by counterflow. The presence of the lectins LcH-A and LcH-B, as well as a complex formed by the two proteins (middle band[42]) in the stack is documented by polyacrylamide gel (PAG) IEF analysis (Fig. 7b).

We have shown during this preliminary investigation of automated RITP that the application of counterflow to the moving steady state system has the capacity to

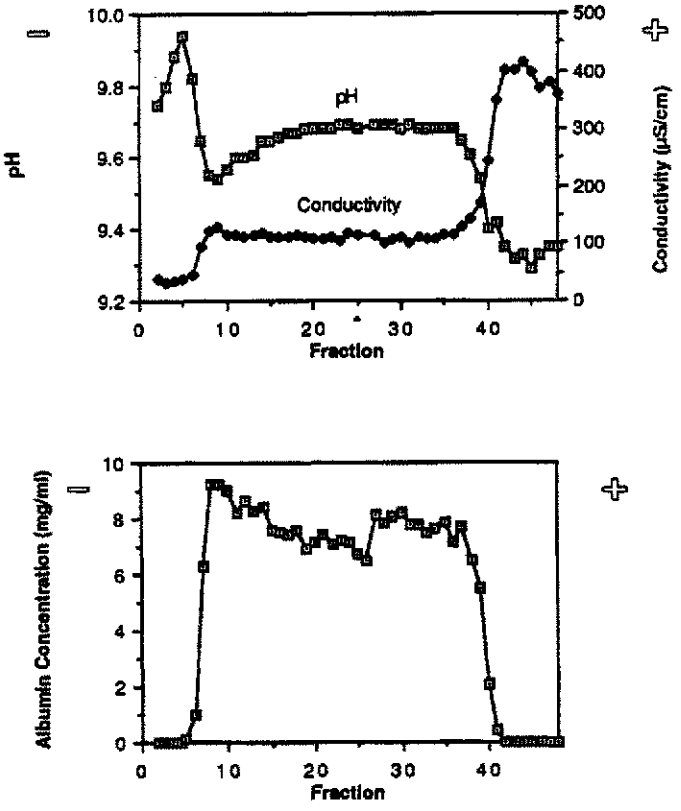

Fig. 6. Anionic RITP of 2880 mg bovine albumin with 5 m*M* hydrochloric acid adjusted with ammediol to pH 9.17 as the leading electrolyte. A 60 m*M* solution of EACA with ammediol at pH 10.44 was used as the catholyte and 15 m*M* hydrochloric acid with ammediol was employed as the anolyte. A constant current of 100 mA was applied. The total voltage at collection time was 1975 V.

move the boundary to any position in the cell which is desired. This capacity is very important for large scale processing of proteins. Careful control of the counterflow applied (or applying constant counterflow and varying the voltage applied) with the computer and an interfaced sensor makes it possible to immobilize the front. This process may extend the effective separation distance to any desired length (essentially infinite) without changing the physical dimensions of the separation chamber. When the migrating steady state has been attained and immobilized at the desired position, any further addition of sample will cause an increased amount of protein in each zone.

RITP offers several advantages to the current modes of preparative electrophoresis, the primary advantage being scale. In our preliminary experiments the loading factor was on the gram scale per processing hour which is a tremendous increase compared to the scale of separations currently obtained in gels. This scale is similar to that reported with CFITP methods[29]. Further research will reveal both the maximum processing rate and the resolution improvement due to recirculation. Many proteins suffer a decrease in activity as well as precipitation after long periods of exposure to

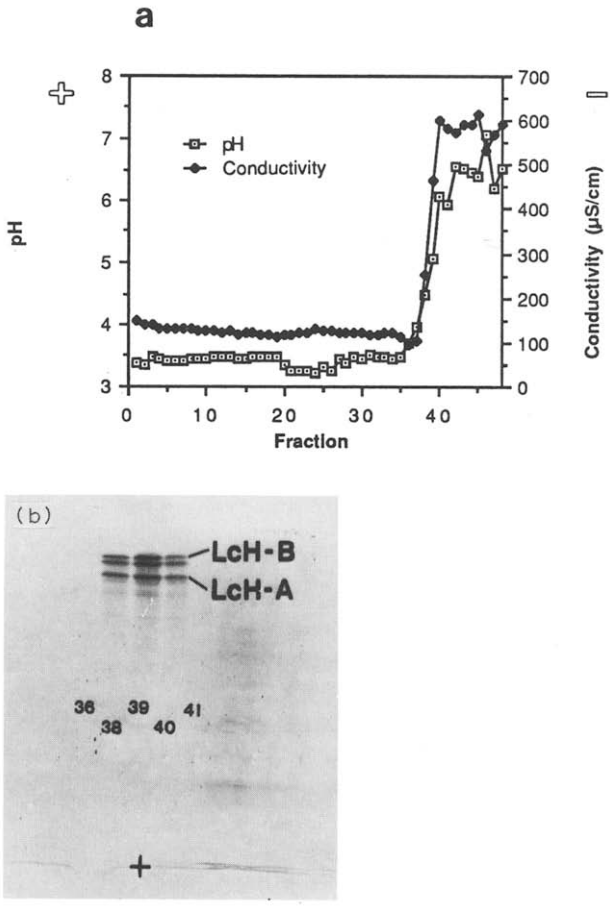

Fig. 7. Cationic RITP of 88 mg lentil lectins prepurified by electrophoresis in the Biostream separator[42]. The leading electrolyte was 10 m*M* potassium acetate, 50 m*M* acetic acid was the anolyte. A constant current of 100 mA was applied. The pH and conductivity profiles after collection (80 min of current flow with 13 min of counterflow) are depicted (a) as well as PAG IEF gel analyses of selected fractions in a pH 3.5–10 ampholine gradient (b).

their isoelectric point which makes preparative IEF a less attractive methodology when considering these proteins. Also, ITP usually does not dilute the sample as is the case with zone electrophoresis.

For complete automation of RITP the computer would have to follow (i) the attainment of the steady state by having arrays of universal and specific sensors, (ii) the monitoring of the position of the front edge of the zone structure and (iii) the activation of counterflow if the leading boundary passes a specified position along the separation axis or of changes of the applied current. Having a sensor specific to the product to be purified would further permit the on-line control of its condensation and recovery. The potential for full computer control of this electrophoretic method may represent a new acceptability of this method as a unit operation for the emerging biotechnology industry. Two different RITP processing procedures, batch and con-

tinuous, are possible. (1) Batch-wise processing: sample injection occurs into the leading electrolyte near the membrane towards the terminator electrode compartment seconds before power application. When the leading boundary passes a specified position bulk counterflow is activated which retards or even stops the further advancement of the front and processing is continued until full separation is achieved. Collection of the separated proteins may proceed in two ways; first bulk collection of the entire separation chamber into several discrete fractions by the use of a multichannel valve to switch all of the recycling channels to a collection apparatus. The second method of collection is by electrophoretic elution; the counterflow is turned off and the entire stack will migrate isotachophoretically to a specific channel which is connected to a computer controlled fraction collector. (2) Continuous operation: a continuous mode of operation can be achieved in which sample is continuously infused, the individual components come to rest at their appropriate position in an ITP stack held stationary by counterflow and a particular product of interest is steadily withdrawn from the appropriate channels.

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