Journal of Chromatography, 545 (1991) 315–329
Elsevier Science Publishers B.V., Amsterdam

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Recycling and screen-segmented column isotachophoresis, two free-fluid approaches for fractionation of proteins

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ABSTRACT

Recycling and screen-segmented column isotachophoresis (ITP), two approaches for the milligrams to grams preparative-scale purification of proteins, are discussed and compared. Recycling ITP was performed in a recycling free-flow focusing apparatus. In this process, fluid flows rapidly through a narrow channel and the effluent from each channel is reinjected into the electrophoresis chamber through the corresponding input port. The residence time in the cell is of the order of 1 s per single pass, which does not allow complete separation, so recycling is essential to attain the steady state. Immobilization of the advancing zone structure is obtained via a controlled counterflow. Thirty fractions of about 4 ml each are obtained. Column ITP was executed in a Rotofor apparatus and in a similar column operated vertically and without rotation. These instruments feature a screen-segmented annular separation space with twenty subcompartments of about 2 ml each. With both approaches, the collected fractions were analysed separately for conductivity, pH and UV absorbance. Selected fractions were characterized by analytical electrophoretic methods. Examples presented include the cationic and anionic ITP behaviour of model proteins, including bovine serum albumin, ovalbumin and ribonuclease A, and the ITP removal of the major impurities from a commercial ovalbumin sample. These examples revealed that the screen-segmented column is suitable for ITP protein purification and operates optimally in a horizontal rotating mode and without internal cooling. The recycling experiments showed that counterflow improves separation and the steady-state patterns are dependent on the fluid layer thickness in the separation cell but, with a given gap, essentially independent of applied current and recycling pump rate.

INTRODUCTION

Isotachophoresis (ITP) is both an attractive purification method which has never been fully characterized [1] and a promising analytical tool for low- and high-molecular-mass components [2]. We are attempting to establish the most suitable free fluid approach for ITP of proteins on the gram scale. So far we have accumulated experience in recycling [3,4] and continuous fow ITP [4] and constructed a mathematical model for the prediction of the ITP behaviour of proteins [5,6].

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Key factors in the design of preparative free-fluid ITP instruments are convective fluid stabilization, temperature control and sample fractionation and detection. Many designs have been discussed (for an overview, see refs. 3 and 7), with recycling and continuous flow ITP currently being the most promising approaches. Monofilament plastic screens placed in the current path were found to be effective for fluid stabilization in recycling [8], rotating [9,10] and vertical column [10] isoelectric focusing. No report was found describing the use of screen segmentation for preparative free-fluid ITP of proteins. Therefore, we decided to explore the potential of using the screen-segmented Rotofor apparatus [9] in various operational modes, including horizontal and vertical chamber arrangements. The ITP fractionation of various model proteins and the purification of ovalbumin in a cationic ITP configuration are described. Data from the Rotofor column were compared with those obtained by performing ITP in a recycling free-flow focusing (RF3) apparatus which was modified for recycling ITP (RITP) via incorporation of an optical boundary sensor in one of the recycling loops and a continuously operating counterflow of leader. The impact of the fluid layer thickness of the RITP separation cell on protein zone formation and fractionation is also discussed. A careful characterization and comparison of the two preparative free-fluid ITP approaches is the topic of this paper.

EXPERIMENTAL

Vertical column isotachophoresis

For ITP experiments in a vertical column, a screen-segmented column identical with that in the commercial Rotofor cell (Bio-Rad Labs., Richmond, CA, U.S.A.) (see below) was used. This cell features an annular chamber with a cooling finger in the centre, polyester screen segmentation and a total column volume of about 52 ml. The separation chamber and electrode vessels were separated by dialysis membranes made from Spectrapor tubing (Spectrum Medical Industries, Los Angeles, CA, U.S.A.). In order to prevent the penetration of electrode reaction products into the column, electrode chambers were modified for flow-through operation and electrode buffers were recirculated from 1-l volume vessels by a peristaltic pump (Vario-Perpex; Werner Meyer, Lucerne, Switzerland) at a flow-rate of 35 ml/min. Before the experiment, the whole separation column (all twenty segments) was filled with the leading electrolyte. Leader from the first segment (whose one wall is formed by the membrane) was removed and the segment was filled with the terminator. Then the electrolyte of the second segment was replaced with the sample dissolved in 2 ml of the leading electrolyte. Immediately thereafter the column was closed, brought to an upright position and the current was applied.

After a run, power was typically disconnected and recycling of the buffers was ceased, prior to collection of fractions using the Rotofor fraction collection box. With this approach the formation of ITP protein zones was studied in both downward and upward migration directions. For the latter configuration, on-line fractionation by continuous protein elution towards the column top was also investigated. For that process, leading electrolyte was pumped (0.6 ml/min) through the second to last segment using a syringe pump (Model 355; Sage Instruments, Cambridge, MA, U.S.A.) equipped with a 60-ml syringe and the effluent was monitored with a Uvicord detector (Model 2158; LKB, Bromma, Sweden) and a high-performance liquid chromatographic flow cell.

Horizontal column isotachophoresis

ITP experiments in the rotating screen-segmented column were performed in the commercial Rotofor apparatus. To increase the rotational speed from about 1 to 4.5 rpm, its motor was replaced with a Model AB 3006-005 (Hurst; Princeton, IN, U.S.A.). Further, the ion-exchange membranes used for isoelectric focusing were replaced with dialysis membranes (see above). The disturbing effects of electrode reaction products were eliminated by filling the electrode chambers with ten times more concentrated electrolytes than used in the separation compartment (in cationic runs, both electrode chambers were filled with 0.1 *M* acetic acid). The application of the sample was performed in the same way as described for the vertical column. Otherwise the experiments were executed according to the manufacturer's specifications for isoelectric focusing.

Recycling isotachophoresis

A prototype of a commercial, recycling free-flow focusing apparatus (Model RF3, Protein Technologies, Tucson, AZ, U.S.A.; distributor, Rainin Instrument, Woburn, MA, U.S.A.) was used for all experiments. This instrument was modified for ITP as described previously [3]. The RF3 instrument is equipped with a built-in power supply for operation at constant voltage, current or power (1500 V, 400 mA, 200 W). Its processing compartment features the separation cell, two electrolyte reservoirs, a multi-channel peristaltic pump, a heat-exchange unit, a 30-channel pulse damper/bubble trap and a 30-tube fraction collector. The total processing volume is about 130 ml. Throughout this work, separation cells of 20 cm length and 4 cm width (made from thick slabs of acrylic) having fluid layer thicknesses of 0.75, 0.5 and 0.25 mm and providing 30 fractions each were used. The outlet temperature was monitored by a sensor located in the tubing bundle between the separation cell and heat-exchange reservoir. Cooling of the system was achieved by attaching an external thermostatic circulator (2219 Multi Temp II; LKB) to the heat-exchange reservoir. The temperature of the cooling fluid (20% ethylene glycol in water) was -2 to 5°C. Typically the fluid temperature within the cell increased to about 15 and 20°C with a recycling pump rate of 30 and 15%, respectively.

For operation in the ITP mode, the electrolyte chambers were separated from the separation channel by dialysis membranes which, for better stability, were backed up by two layers of chromatographic paper (3MM CHR; Whatman, Maidstone, UK). Electrode buffer reservoirs of 250 ml (RF3 standard is 60 ml) were used and filled with buffers of 10-fold higher concentration than employed within the separation cell. The sample was injected in channel 2, which is near the terminator electrolyte chamber, and the vent was moved to channel 14. The advancing protein boundary was detected by a Model 2138 Uvicord S detector (Pharmacia–LKB, Uppsala, Sweden) with a 277-nm filter. The detector was inserted into the recycling loop of channel 26 (near the leading electrolyte chamber). The detected signal was registered as a function of time on a strip-chart recorder (Series 1200; W + W Scientific Instruments, Basle, Switzerland). The counterflow inlet and outlet were placed in channels 30 and 1, respectively. The counterflow was generated using a low-pulse peristaltic pump (Minipuls 3; Gilson Medical Electronics, Middleton, WI, U.S.A.) together with a laboratory-made pulse damper and bubble trap, and was regulated manually.

All the experiments were performed in a batch mode of operation according to

the manufacturer's instructions. The separation cell was filled with the leading electrolyte. The multi-channel peristaltic pump was set to a pumping rate of 15–30% and the recycling of the electrolytes was started. The sample, dissolved in a maximum of 10 ml of the leading electrolyte and filtered through a 0.45-μm membrane syringe filter, was slowly and carefully injected into the electrolyte stream inside the separation cell. After sample injection, power was applied at a constant current of 34–50 mA. In experiments without counterflow, collection of the 30 fractions occurred immediately after the front protein boundary was recorded via an increase in absorbance (277 nm) within channel 26. In all other experiments, on the occurrence of the absorbance change the counterflow was activated and manually adjusted so as to maintain a constant absorbance level. The ITP zone structure was thereby immobilized. Typically the counterflow pumping rate did not exceed 1.5 ml/min for the anionic and 3.0 ml/min for the cationic systems.

Analysis of collected fractions

For pH measurements a Model 720 pH meter and a Ross Model 8103 SC pH electrode (both from Orion, Cambridge, MA, U.S.A.) were used. The conductivity was measured with a Model 101 conductivity meter (Orion) equipped with a Model PW 9510/65 cell (Philips, Eindhoven, Netherlands). The absorbance was measured at 280 nm in a Lambda 15 UV–VIS spectrophotometer (Perkin-Elmer, Überlingen, Germany). In some instances the fractions containing the protein zones had to be diluted 10-fold and for presentation of the data the absorbance values were multiplied by ten. Selected fractions were also analysed by capillary ITP using a Tachophor 2127 analyser (LKB). This instrument was equipped with a 28 cm × 0.5 mm I.D. PTFE capillary, and a conductivity and UV detector (277-nm filter) at the column end. The same electrolyte systems as used for preparative ITP were employed. The measurements were performed at a constant current of 150 μA.

The staining of bovine serum albumin was performed by adding 5 μl of bromophenol blue solution (7.5% in 0.1 *M* sodium hydroxide) to each 75 mg of protein. The bovine serum albumin plateau concentration was calculated from a measured standard solution containing also bromophenol blue.

Chemicals

All chemicals were of research-grade purity. Bromophenol blue, ribonuclease A, γ-amino-*n*-butyric acid, 2-amino-2-methyl-1,3-propanediol (ammediol) and β-alanine were obtained from Sigma (St. Louis, MO, U.S.A.), albumin from chicken egg (crystallized five times, 11840/D8) from Serva (Heidelberg, Germany), bovine serum albumin from Fluka (Buchs, Switzerland) and potassium acetate, formic acid and acetic acid from Merck (Darmstadt, Germany).

RESULTS AND DISCUSSION

Test of performance

In order to compare the performances of the various instrumental approaches, *i.e.*, their ability to run under ITP conditions and to provide ITP zones, all experiments reported in this section were made with the same anionic model system, an ITP configuration which was recently described theoretically and validated by capillary

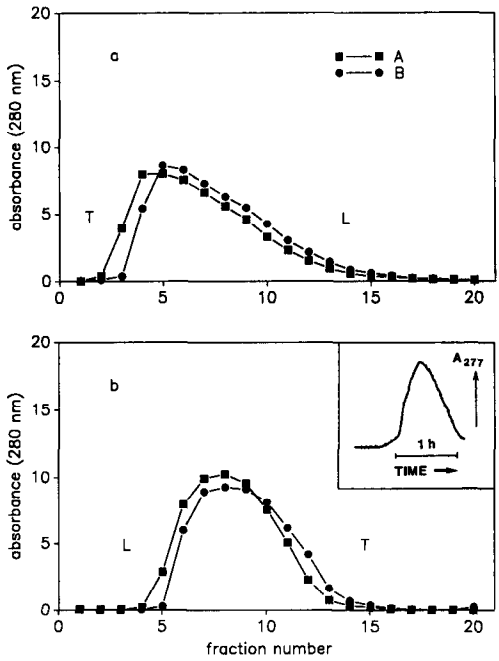

Fig. 1. Formation of a BSA zone (220 mg of protein) in vertical column ITP with downward [(a) constant 8 mA for 60 min] and upward [(b) 8 mA for 100 min] migration. Graphs A and B represent the harvested protein distributions without and with internal cooling, respectively. L and T refer to the positions of leading and terminating zones, respectively. The voltages at collection time were 400 and 900 V, respectively. The plotted absorbance values represent measurements for 10-fold diluted fractions which were multiplied by ten. The graph depicted in the inset in (b) represents the detected BSA zone (75 mg) after elution in a run with upward migration at a constant 10 mA and internal cooling. The peak maximum was detected after 230 min (absorbance range was 2.0).

ITP [6]. It consists of 0.01 *M* formic acid and ammediol (pH_L = 9.0) as the leader, 0.01 *M* β-alanine and ammediol (pH_T = 9.5) as the terminator and bovine serum albumin (BSA) stained with bromophenol blue as the sample.

In the vertical column two running modes were tested, *viz.*, migrating the ITP system upwards (against gravity) and downwards, both either with or without cooling by circulating tap water (15°C) through the cooling finger of the column. Fig. 1 shows results in the form of BSA absorbance profiles, as determined by measuring the UV absorbance of the collected fractions. In all four experiments the upper BSA boundary was sharper than the lower transition. As was observed visually, the disturbance at the lower zone boundary is caused mainly by sedimentation of drops of the dense protein zone into the lower buffer [leader in (a), terminator in (b)]. Obviously, in upwards migration the disturbing effect is counteracted by electromigration which proceeds in the opposite direction, the BSA zone thus being continuously restored. In downwards migration there is no such effect and therefore the resulting BSA profile is much broader (Fig. 1a). The monitored BSA absorbance profile after continuous elution from the top is depicted as the inset in Fig. 1b. There is a good correlation between zone shape, but the elution process is slow and dilutes the sample.

Another important but less disturbing effect is thermal convection of the liquid within each segment. Joule heating of the electrolytes leads to the formation of temperature gradients. With cooling by circulating water through the cooling finger, the most pronounced temperature gradient is formed towards the wall of this finger, forcing the contents of each segment to circulate so that the liquid flows downwards at the cooling finger wall (at the inner wall of the annular column). Without internal cooling, the driving temperature gradient is formed towards the outer column wall. In this instance the circulation of the liquid in the segments is both reversed and decreased. These fluid flow patterns were observed visually by having small amounts of dyed BSA within the leader/terminator boundary. Operation without internal cooling produces less thermal convection, which results in slightly narrower BSA concentration profiles (Fig. 1b). Based on the above observations, it can be concluded that the best result was obtained by migrating the system upwards and operating it without cooling.

Having a horizontal arrangement of the annular and screen-segmented column without rotation around the separation axis, gravity was found to cause protein slumping (data not shown) in a similar way to that previously observed for isoelectric focusing [10]. Fig. 2 shows the results of running the same anionic ITP system in the horizontal rotating arrangement. Experiments showed that an increased rotation

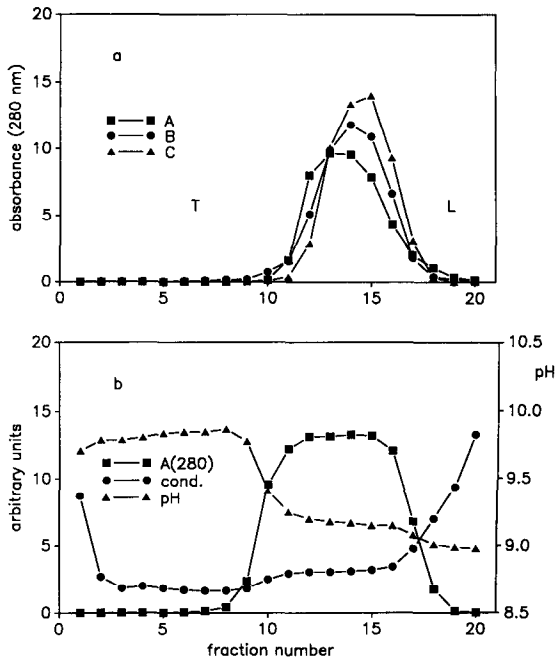

Fig. 2. Formation of a BSA zone in horizontal column ITP (Rotofor) with (a) 230 mg and (b) 480 mg of protein. The graphs in (a) were obtained under the following conditions: (A) 1 rpm, with internal cooling; (B) 4.5 rpm, with cooling; (C) 4.5 rpm, without cooling. The run depicted in (b) was executed at 4.5 rpm without cooling. A constant current of 15 mA during 50 min of electrophoresis time was applied in all instances. The units for the conductivity are 0.01 S/m. The plotted absorbance values represent measurements for 10-fold diluted fractions which were multiplied by ten.

speed (from 1 rpm as provided with the commercial Rotofor to 4.5 rpm) improved the formation of a BSA ITP zone (*cf.*, graphs A and B in Fig. 2a); therefore, all further experiments were performed under these conditions. Comparison of graphs B and C also shows that some improvement was reached when operating without internal cooling. As the last experiment (rotating horizontal mode, 4.5 rpm, without cooling) gave the best result for the screen-segmented column, an additional experiment was made with a larger amount of BSA. With 480 mg of BSA a good ITP zone profile with reasonably sharp boundaries and a well developed concentration plateau corresponding to a steady-state BSA concentration of 20 mg/ml was obtained

A large number of recycling ITP experiments showed that BSA establishes ITP zones under a relatively wide range of experimental conditions and that they compare well with computer-simulated data [3,4]. Fig. 3a shows a typical profile obtained by operating at a constant current of 50 mA and with a fluid layer gap of 0.75 mm. The relatively short path length in the separation cell brought up the question of whether the sample zone can reach a steady state within the time of the experiment. Fig. 3b shows the result with application of a counterflow otherwise executed with the same conditions as used for Fig. 3a. The two experiments appear to be at the steady state when judged by the zone shape. However, careful inspection of the two protein profiles reveals that the counterflow caused an additional sharpening, resulting in a narrower zone with an increased BSA plateau concentration [26 instead of 22 mg/ml (Fig. 3a)]. Fig. 3c illustrates that with variation of electric current and/or recycling rate, small changes in zone profiles were obtained. The plateau BSA concentrations varied from 26 mg/ml (current 50 mA, flow-rate 30%, profile A and Fig. 3b) to 28 mg/ml at 34 mA and a 30% pump rate (profile B) and 30 mg/ml at 34 mA and a 15% pump rate (profile C).

Test of separation power

The second part of the investigations was aimed at establishing whether the instrumental arrangements are suitable for running multi-zone ITP systems involving proteins. For all the experiments presented here, a cationic model system was selected consisting of 0.01 *M* potassium acetate and acetic acid ($pH_L = 4.75$) as the leader and 0.01 *M* acetic acid as the terminator. Ovalbumin (OVA) and ribonuclease A (RNA) were used as the model protein pair. Preliminary investigations by analytical ITP showed that these proteins are separated well in the given electrolyte system and that γ-aminobutyric acid (GABA) can be successfully used as a spacer.

Fig. 4 shows the results obtained in the screen-segmented column (all without cooling). Small amounts of both proteins were sampled together with a large amount of spacer; this amount was selected so that the adjusted spacer zone filled about seven column segments. Fig. 4a and b show a comparison of two runs in the vertical column with downwards and upwards migration, respectively. It is seen that, in contrast to the anionic system, downwards migration provides much better results. Fig. 4c shows the profiles obtained in the rotating horizontal mode (4.5 rpm); here the result is comparable to that in Fig. 4a. Cooling was found to slightly deteriorate the separation. It can be concluded for this type of column that RNA and OVA are concentrated at the front and rear boundaries of the well developed spacer zone, respectively (as is indicated by both the UV absorption and pH profiles). Although there is no baseline resolution, the two proteins are well separated. This was also confirmed by capillary

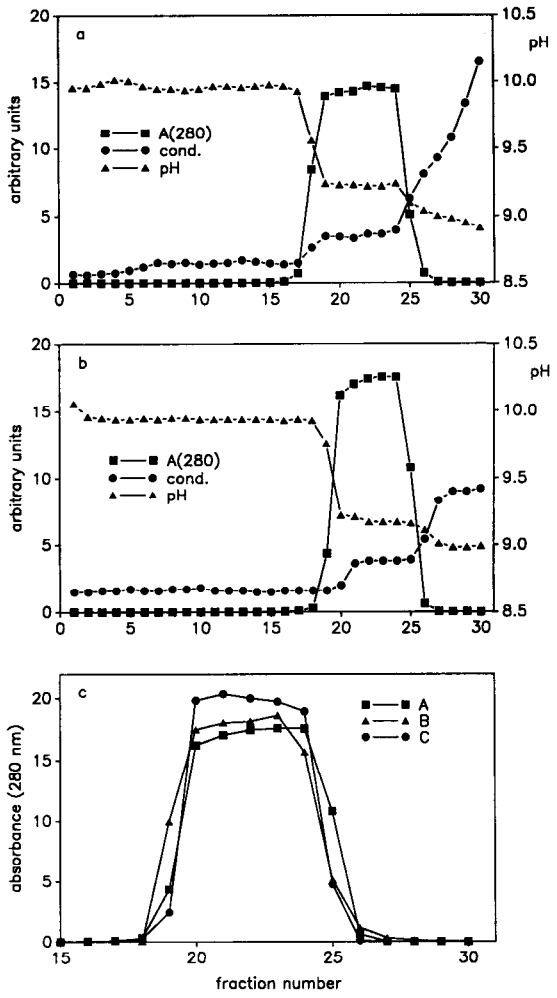

Fig. 3. Recycling ITP data for 750 mg of BSA run without a counterflow [(a) 53 min run time] and with 56 min of counterflow [(b) 105 min run time]. The current was a constant 50 mA and the pump rate was 30% in both instances. The total voltages at the collection time were (a) 1062 V and (b) 804 V. In the latter instance the voltage dropped from 1016 to 804 V during the counterflow period. The processing charges were (a) 159 C and (b) 315 C. (c) Counterflow data with a constant (A) 50 mA, (B) 34 mA and (C) 34 mA together with a recycling pump rate of 30, 30 and 15%, respectively. The total processing time intervals (counterflow periods) were 105 (56), 135 (64) and 122 (49) min, respectively. The final (values at time of counterflow activation) voltages were 804 (1016), 445 (503) and 394 (383) V and the total processing charges were 315, 275 and 249 C, respectively. In all instances a cell with a 0.75-mm gap was employed. The scales are the same as in Fig. 2b.

ITP analyses of the collected fractions (Fig. 5). In both experiments the major RNA fractions were essentially free from OVA whereas the major OVA fractions were found to have a residual RNA content. This was much more pronounced in the upwards migration experiment (Fig. 5b). The result of fractionation via continuous elution after

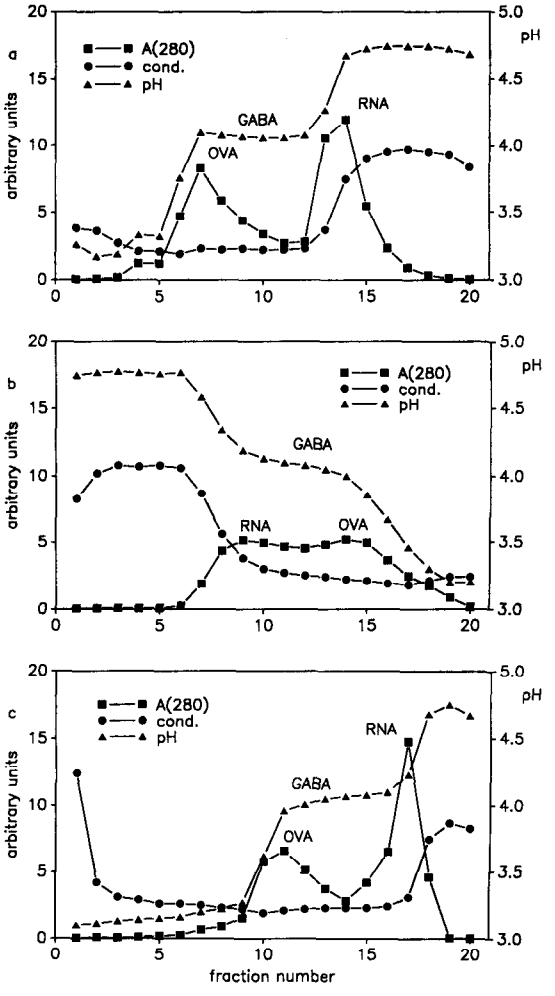

Fig. 4. Column ITP of RNA and OVA with GABA as spacer in (a) a vertical arrangement with downward migration, (b) a vertical set-up with upward migration and (c) in the Rotofor at 4.5 rpm. All experiments were performed without internal cooling. The sampled amounts and electrical conditions were as follows: (a) 12.6 mg OVA, 12.5 mg GABA, 13.1 mg RNA, 5 mA for 150 min, 170 V final; (b) 9.4 mg OVA, 11.7 mg GABA, 10.2 mg RNA, 5 mA for 150 min, 160 V final; (c) 12.9 mg OVA, 9.4 mg GABA, 11.0 mg RNA, 15 mA for 60 min, 390 V final. The units for the conductivity are 0.01 S/m. Absorbance values from undiluted samples were multiplied by ten.

upward migration is depicted in Fig. 6. This approach was found to substantially elongate a run (with the advantage of having an increased separation capacity), but with the disadvantages of sample dilution and partial remixing in the elution chamber. Fig. 7 shows the result of a similar run in the recycling apparatus with counterflow. Here a much longer spacer zone could be generated and the separation of the two proteins is almost complete. For this example, the peak concentration of OVA was

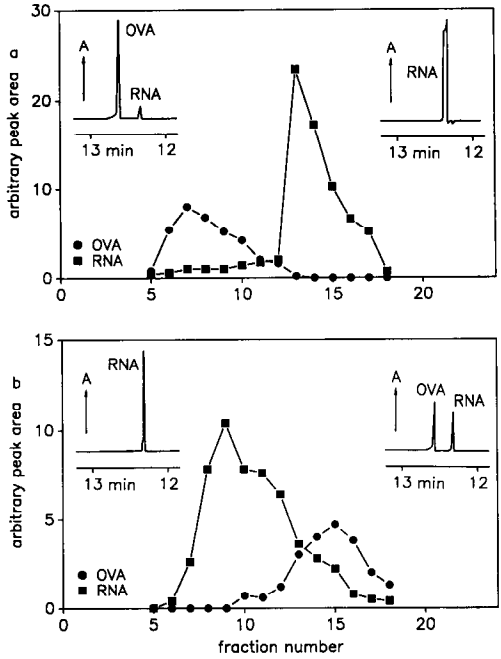

Fig. 5. Capillary ITP analysis data for the separation depicted in (a) Fig. 4a and (b) Fig. 4b. For each fraction and protein the peak area of the appropriate UV signals was plotted. The insets depict the Tachophor UV (277 nm) responses of fractions corresponding to the absorbance maxima [(a) fractions 7 and 14; (b) fractions 9 and 14]. Volumes of 2 μl of undiluted fractions were injected and analysed with a constant 150 μA.

found to be 11 mg/ml and the apparent (see below) RNA content was determined as 9.7 mg/ml. The RNA plateau concentration was 12.6 mg/ml (data not shown).

We could show by analytical ITP (data not shown) that the RNA fractions from

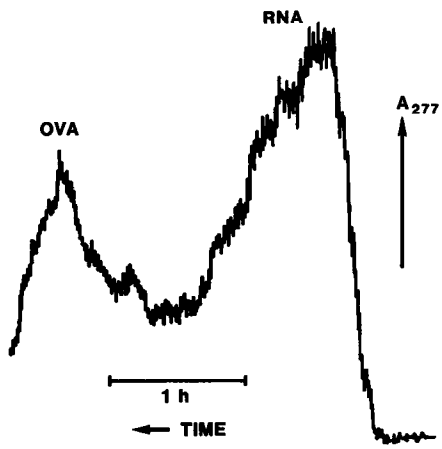

Fig. 6. Elution data (absorbance range 0.1) of an experiment with 6 mg of RNA, 51 mg of GABA and 5.5 mg of OVA. The current was 12 mA for the first 65 min and 10 mA during sample elution. The RNA and OVA peaks were detected after 95 and 115 min, respectively.

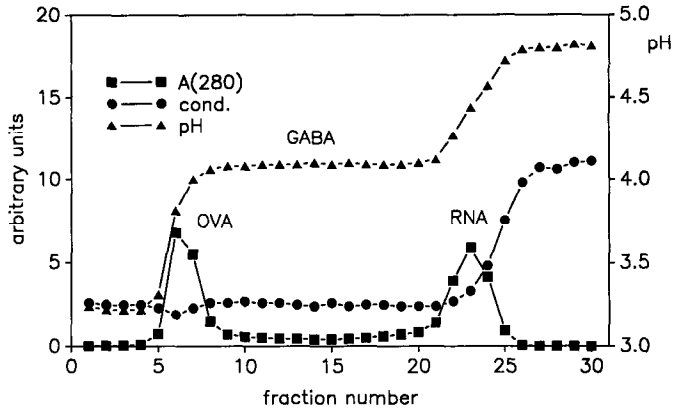

Fig. 7. Recycling ITP of 105 mg of RNA, 42 mg of GABA and 125 mg of OVA. The current and the pump rate were 50 mA and 30%, respectively. Counterflow, activated after 53 min (460 V), was applied for 47 min (511 V final). The charge applied was 300 C. Conductivity and absorbance scales are identical with those given in Fig. 2b.

the above-described experiments also contained some major impurities from the commercial ovalbumin sample used. We therefore adopted the problem of purification of ovalbumin by removal of two of its major impurities, lysozyme and conalbumin, to give the second model example in this part of the study. Fig. 8a depicts the result obtained with the rotating segmented column (operated without cooling at 4.5 rpm). Similar data were obtained in the recycling apparatus. As shown in Fig. 8b and c, operation with a counterflow (Fig. 8c) provides better results than operation without a counterflow (Fig. 8b). The OVA peak concentration (fraction 16, Fig. 8c) was found to be 25 mg/ml. The effectiveness of the recycling ITP purification procedure is indicated by capillary ITP analyses of individual fractions. Fig. 9a and b show the original ovalbumin sample analysed without and with GABA as spacer, respectively, illustrating a relatively high content of impurities migrating in front of the GABA zone (predominantly lysozyme and conalbumin, as stated by the supplier). Fig. 9c shows the analysis of a fraction containing mainly OVA and some residual impurities. In Fig. 9d the analysis of a fraction with maximum impurity concentration is shown

The present model system was further employed to investigate the impact of other parameters. Of great interest was the question of how the separation performance is affected by the thickness of the liquid layer in the RITP separation cell. Fig. 10 depicts a comparison of the absorbance profiles from OVA purification having cells with thickness 0.75 (as in all previous runs), 0.50 and 0.25 mm. No significant influence of the cell gap was observed when operating without a counterflow (Fig. 10a). The application of a counterflow, however, was found markedly to dilute and broaden the OVA zone with decreasing cell thickness (Fig. 10b). With no counterflow separation was incomplete and comparable in all three instances, whereas with a counterflow the resolution was found to increase with decreasing gap size. Variation of the electric current and the recycling pump rate did not affect the separation in a given cell assembly, as is shown in Fig. 10c (data for a 0.25-mm cell). These results demonstrate that identical zones (*i.e.*, steady state) are obtained with

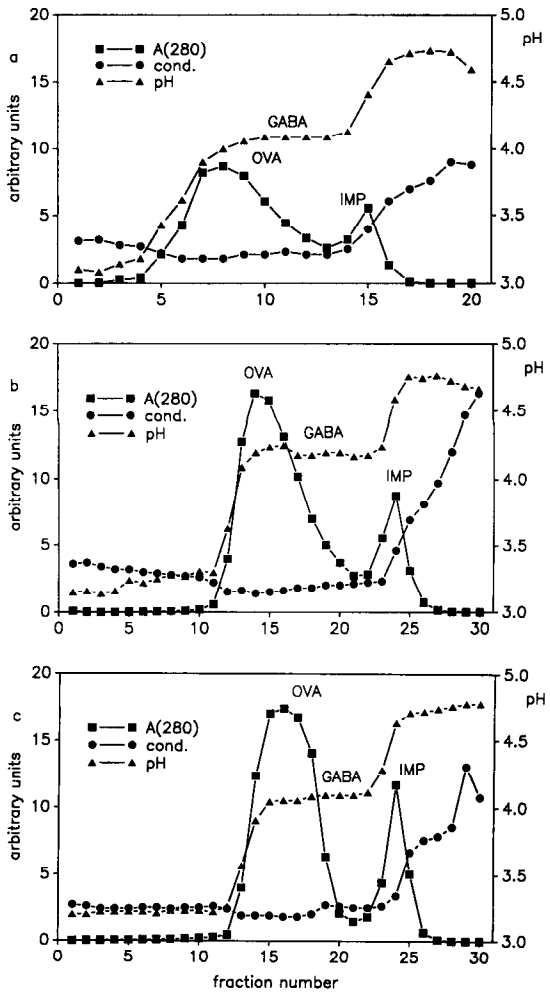

Fig. 8. (a) ITP of 310 mg of OVA and 9.1 mg of GABA in the Rotofor (no cooling) at 4.5 rpm. A constant 15 mA was applied for 60 min (final voltage 430 V). Recycling ITP data for 710 mg of OVA and 14 mg of GABA (b) without counterflow (60 min run time) and (c) after 52 min of counterflow (112 min run time). In both instances the current and the recycling pump rate were a constant 50 mA and 30%, respectively. The total voltages at the collection time were 510 and 463 V (450 V at counterflow activation), respectively. The processing charges were 180 and 346 C, respectively. Scales are identical with those in Fig. 2b.

a relatively wide range of parameters; 300 C were apparently sufficient to reach steady state. This is considered to provide confirmation that the three runs depicted in Fig. 10b are at the steady state. The internal volumes of the three cells (6, 4 and 2 ml) are small compared with the total volume of the recycling channels (*ca.* 120 ml) and are therefore not considered to be responsible for the change in the zone patterns. It is shown in this work that counterflow, at a given gap size, influences the steady-state distribution. Further tests are needed to elucidate the impact of counterflow on zone formation at various gap sizes.

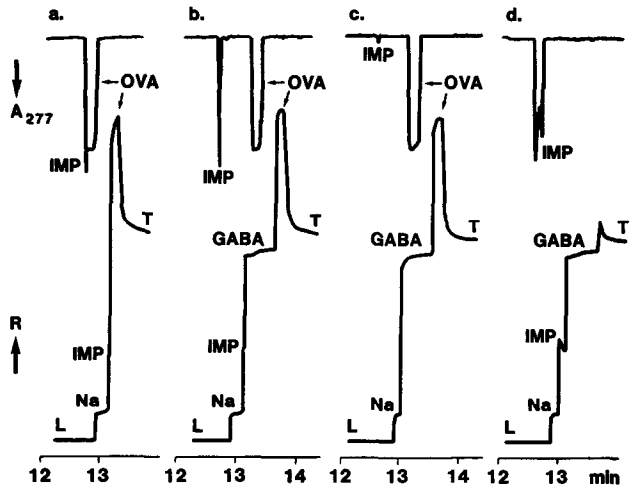

Fig. 9. Capillary ITP analyses of (a) commercial OVA, (b) OVA with GABA and (c, d) two fractions of a typical OVA/GABA recycling ITP run which were spiked with GABA. Samples of 1 μl of a 24 mg/ml OVA solution (a, b) or of undiluted fractions (c, d) were injected. For runs b–d 0.5 μl of a 4.2 mg/ml solution of GABA was co-administered. The lower and upper graphs depict the conductivity (expressed as increase in resistance, R) and UV absorbance at 277 nm, respectively. A constant current of 150 μA was applied. IMP: impurity.

CONCLUSIONS

ITP of proteins in screen-segmented columns, reported here for the first time, is an attractive approach for the fractionation of proteins in the milligrams to grams range. These investigations revealed that (i) in all operational modes ITP zone formation was slightly better without internal cooling (*cf.*, Figs. 1 and 2); (ii) having a vertical column downward migration provided better ITP zones in one instance (Fig. 4a and b) whereas upward migration was better for another (Fig. 1a and b); (iii) collection of all twenty fractions at once gave better results than fractionation via continuous elution at one column end, for which the experiments became very long and fractions were diluted with leader and partially mixed (Figs. 4 and 6); and (iv) having a horizontal column rotate around its separation axis was found to be essential and rotation at 4.5 rpm was shown to be preferred to 1 rpm (Fig. 2). For all systems investigated, the horizontal arrangement with 4.5 rpm and without internal cooling provided the best results.

The recycling ITP data demonstrated that (i) a counterflow was required for the purification of proteins (Figs. 8a and b, and 10a and b); (ii) a counterflow changed the steady-state protein profile (Fig. 3); and (iii) steady-state profiles were dependent on the gap size but not markedly influenced by the applied current or recycling pump rate (Fig. 10). The last fact provides an explanation for the previously reported difference between the plateau BSA concentrations found for recycling and continuous flow ITP [4]. This effect, however, cannot be explained with the current mathematical ITP models. The impact of gap size on the steady-state distribution is another phenomenon that remains to be explained from a theoretical point of view.

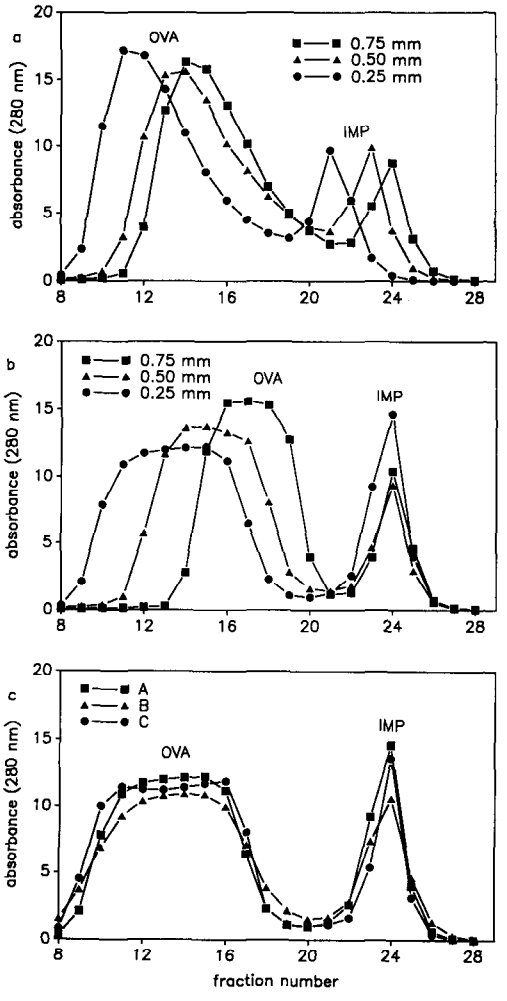

Fig. 10. Recycling ITP absorbance data of 710 mg of OVA and 14 mg of GABA (a) without and (b) with counterflow as functions of the gap size. The pumping rate was 30% in all instances. Without counterflow the total charges were 180 C (conditions: 50 mA, 60 min, 510 V final), 159 C (34 mA, 78 min, 623 V) and 159 C (34 mA, 78 min, 1095 V) for the 0.75-, 0.5- and 0.25-mm cells, respectively. With counterflow the charges were 346 C (conditions: 50 mA, 463/450 V final/at counterflow activation), 380 C (50 mA, 798/730 V) and 534 C (50 mA, 1561/1604 V), respectively. The durations of counterflow were 52, 60 and 120 min, respectively. (c) Recycling ITP absorbance data for 720 mg of OVA and 14 mg of GABA with counterflow in the 0.25-mm gap cell. The applied charges were (A) 534, (B) 308 and (C) 316 C, the constant currents were 50 mA (conditions: 1560/1604 V final/at activation of counterflow), 34 mA (1052/1091 V) and 34 mA (1050/1092 V), the recycling pump rates were 30, 30 and 15%, counterflow was activated after 58, 84 and 85 min and collection occurred after 178, 151 and 155 min, respectively.

Comparison of column ITP and recycling ITP data revealed that a better resolution was obtained with the latter approach (*cf.*, Figs. 4 and 7, Fig. 8). For example, in recycling ITP the load capacity was not completely exhausted in the experiments depicted in Fig. 8b and c. Therefore, the resolution could be enhanced by

employing larger amounts of spacer. In column ITP the protein zone pattern occupied almost the whole column length (Fig. 8a). Recycling ITP runs were executed at *ca.* 330 A/m^2 (cell with 0.75 mm gap; 25–50 W) compared with *ca.* 65 A/m^2 (5–10 W) in the column experiments. The throughputs investigated were all of the order of several hundred milligrams of protein per hour. Although not yet thoroughly investigated, scale-up of recycling ITP should be simpler than for column ITP.

Commercially available OVA contains major impurities which, in a cationic ITP configuration, were found to migrate in front of this protein. Using GABA as a spacer, OVA could be purified in both types of instruments. Analysis by capillary ITP revealed that the major OVA fractions were essentially free from the impurities (Fig. 9). This example constitutes an interesting model system for evaluation of the recycling ITP operational modes and we are therefore planning to study further the removal and purification of these impurities by recycling ITP.

ACKNOWLEDGEMENTS

The generous loan of the Tachophor 2127 and Uvicord 2158 instruments by LKB is gratefully acknowledged. The authors thank the Liver Foundation, Berne, for the purchase of the Rotofor, T. Long for providing the counterflow pulse damper and Ciba-Geigy for the ovalbumin sample. P.G. thanks his employer for granting study leave. This work was supported by the Swiss National Science Foundation.

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