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Continuous separation and purification of proteins by recycling isotachophoresis

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ABSTRACT

The continuous separation and purification of proteins by recycling isotachophoresis (RITP) is described. By operating the RITP fractionation process under counterflow conditions with immobilized sample zones, a configuration is available which allows the continuous feed of crude sample and withdrawal of the purified product. The concept is demonstrated by anionic RITP of bovine serum albumin (BSA) and by cationic RITP purification of ovalbumin (OVA) and lysozyme (LYSO) from a commercial OVA product containing LYSO and conalbumin as major proteinaceous impurities. For the OVA and LYSO purifications, continuous RITP provided a higher sample throughput and comparable product purity compared with batch RITP. In the BSA and LYSO separations the compounds to be purified established their isotachophoretic zones at the front of the sample stack, which represents the simplest example of continuous RITP operation. Continuous purification of OVA as a compound located at the rear of the isotachophoretic zone structure is more complex. With prolonged processing time the removal of proteinaceous impurities which accumulate in the front part of the cell, *i.e.* near the counterflow inlet, is required. Components which do not form their isotachophoretic zone at or near the edges of the zone structures can only be partially purified by continuous RITP. For such compounds a semi-continuous mode of operation in which sample infusion and the withdrawal of purified product are alternated is proposed.

INTRODUCTION

Recycling isotachophoresis (RITP) is a relatively new free fluid approach for preparative isotachophoresis (ITP) [1–6]. In this method the 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 separation cell is of the order of one second per single pass, which does not allow complete separation. Thus recycling is essential to attain a steady state. Immobilization of the advancing zone structure is obtained via a controlled counterflow. RITP has been shown to be an attractive purification method for egg white proteins [6].

In its simplest implementation, RITP is used as a large-scale batch process with a specified amount of sample solution being injected near one end of the separation cell, at the location of the initial interface between the leader and terminator [1–6]. This paper reports a continuous mode of operation in which the 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 channel.

MATERIALS AND METHODS

Chemicals

All chemicals used were of research grade purity. γ-amino-*n*-butyric acid (GABA), 2-amino-2-methyl-1,3-propanediol (ammediol) and β-alanine were from Sigma (St. Louis, MO, USA). Bovine serum albumin (BSA) was purchased from Fluka (Buchs, Switzerland). Ovalbumin (OVA) from chicken egg (5× crystallized, lot No. 11840/D8) and tetrapropylammonium bromide (TPAB) were from Serva (Heidelberg, Germany). Potassium acetate, formic

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acid and acetic acid were from Merck (Darmstadt, Germany).

RITP

The RITP instrument used in this work is the same as described previously [5,6]. Briefly, it consists of a recycling free flow focusing apparatus (Model RF3; Protein Technologies, Tucson, AZ, USA, distributed by Rainin Instrument, Woburn, MA, USA) with modifications for RITP. Throughout this work, a separation cell of 20 cm length and 4 cm width with a fluid layer thickness of 0.75 mm and providing 30 fractions was used. The total processing volume was about 130 ml. The outlet temperature was about 15°C (cooling bath 7.5°C) with a recycling pump rate of 30% and a constant current of 50 mA. 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 2 l (RF3 standard is 60 ml) were used and filled with buffers of ten-fold higher concentration than that used within the separation cell.

The boundary of a sample protein zone was detected by a 2138 Uvicord S instrument (Pharmacia LKB, Uppsala, Sweden) with a 277 nm filter. Its location was dependent on the purification sample (see Fig. 1). 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, USA) together with a laboratory-made pulse damper and a bubble trap. It was regulated manually with registration of the output signal from the boundary detector on a strip-chart recorder. Sample feed and bleed were executed with two independent, low-pulse peristaltic pumps (Vario Perpex, H. J. Guldener, Zürich, Switzerland).

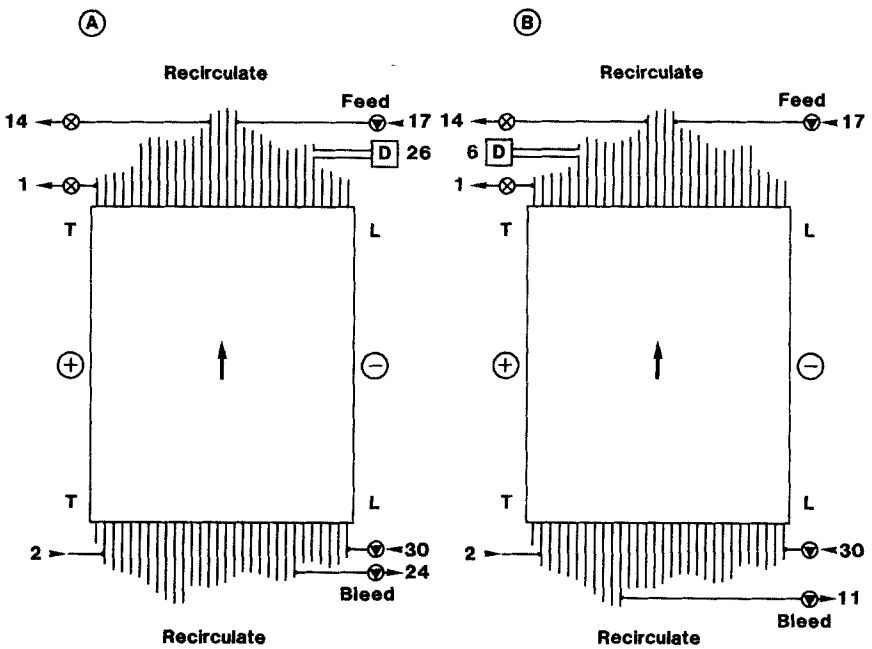

Fig. 1. Schematic diagram of the experimental set-up for cationic RITP. Bolus injection of the sample occurs through inlet 2 and the extra fluid is drained through an outlet in channel 14 (outlet 1 is closed). Counterflow is introduced into channel 30 and drained through outlet 1 (outlet 14 is closed). (A) Continuous purification of a compound at the ITP front with immobilization at channel 26, the location of the boundary detector, D. Sample feed and purified product withdrawal (bleed) are at channels 17 and 24, respectively. (B) Continuous purification of a compound which establishes its ITP zone at the end of the stack. The boundary detector is located in channel 6. Sample feed and purified product withdrawal (bleed) are at channels 17 and 11, respectively. For anionic RITP the polarity must be reversed. L and T refer to leader and terminator, respectively.

These pumps were equipped with 1/10 and 1/20 gears, respectively.

All experiments began with the separation cell filled with the leading electrolyte. The RF3 multichannel peristaltic pump was set to a pumping rate of 30% and the recycling of the electrolytes was started. The sample, dissolved in 10 ml of the leading electrolyte and filtered through a 0.45-μm membrane syringe filter (Nalgene; Nalge, Rochester, NY, USA), was slowly and carefully injected into the electrolyte stream through channel 2. After sample injection, power was applied at a constant current of 50 mA. When the absorbance in the monitoring loop changed, the counterflow was activated to maintain a constant absorbance. The ITP zone structure was thereby immobilized. Typically, the counterflow pumping rate did not exceed 2.0 ml/min. After the establishment of these steady-state conditions, the sample was continuously infused and the purified product withdrawn according to the configurations shown in Fig. 1. At the end of a run, the remaining processing fluid was collected as described for the batch process [5,6].

Analysis of collected fractions

For pH measurements a Model 720 pH meter and a Model 8103 SC ROSS pH electrode (both from Orion Research, Cambridge, MA, USA) were used. The conductivity was measured with a Model 101 conductivity meter (Orion Research) equipped with a Model PW 9510/65 cell (Philips, Eindhoven, Netherlands). The absorbance was measured at 280 nm in a UV–VIS Lambda 15 spectrophotometer (Perkin-Elmer, Überlingen, Germany). The fractions containing high protein concentrations had to be diluted ten times. For presentation of the data the absorbance values were multiplied by ten. Fractions containing conalbumin (CAL) showed a high turbidity and had to be filtered before analysis.

The feed and bleed solutions, in addition to selected fractions at the end of a run, were analysed by capillary ITP using a Tachophor 2127 analyser (LKB, Bromma, Sweden). This instrument was equipped with a 28 cm PTFE capillary of 0.5 mm I.D., as well as a conductivity and UV detector (filter 277 nm) at the column end. The measurements were performed at a constant current of 150 μA. The data were recorded on a two-channel strip-chart recorder or with a data acquisition system consisting of a PC Integration Pack (Version 2.50, Kontron Instruments, Zürich, Switzerland) together with a Mandax AT 286 computer system, or both. This integration pack features two channels for data acquisition, automatic range switching and a dynamic sampling rate allowing a data sampling rate of up to 100 Hz for quickly changing signals. The digitized and computer-stored UV signals of the proteins were integrated (peak area determination), thus providing quantitative data for pure protein zones separated by non-absorbing spacers.

RESULTS AND DISCUSSION

Continuous anionic RITP of BSA

Anionic RITP was performed as reported previously [5] with a leader composed of 10 m*M* formic acid which was adjusted to pH 9 with ammediol. The anodic electrode compartment contained a tenfold higher concentration of the same buffer. A 100 m*M* solution of β-alanine (terminator), adjusted with ammediol to pH 9.5, was used in the cathodic compartment. The RITP set-up used, but with reversed polarity, is shown in Fig. 1A and the sequence of experimental stages is summarized in Table I.

RITP was first performed with 750 mg of BSA, which was dissolved in 10 ml of 0.01 *M* leader and introduced through port 2 into the cell before the application of power. After a run time of 50 min with a constant applied current of 50 mA, the protein front reached the detection loop (channel 26) and counterflow was activated. Immobilization of the BSA zone was achieved and maintained for 25 min at a power application of about 40 W (stage II).

Continuous infusion (feed) of the sample into channel 17 (Fig. 1A), a channel containing adjusted terminating electrolyte (Fig. 2), and the withdrawal of BSA from channel 24 were then started (stage III of Table I). Using computer simulation, the composition of the terminating buffer was calculated to be 7.48 m*M* β-alanine and 33.8 m*M* ammediol (pH 9.77; conductivity 16.6 mS/cm) [7]. To minimize buffer perturbations at the location of sample infusion, the feed solution was composed of this adjusted buffer. The concentration of BSA in the buffer was 8.33 mg/ml and the pH and conductivity were 9.95 and 17.2 mS/cm, respectively. The infusion rate was 1.2 ml/min. BSA was withdrawn at a

TABLE I

EXPERIMENTAL STAGES FOR CONTINUOUS ANIONIC RITP OF 1.5 g BSA

The time represents the time interval of a stage, the charge is the total charge spent during a stage, the voltage is given as the initial and end voltage of a stage, the counterflow values represent average flow-rates and the yield is the total amount of BSA recovered during a stage.

Stage	Time (min)	Charge (C)	Voltage (V)	Counter-flow (ml/min)	Sample feed		Bleed rate (ml/min)	Yield of BSA (mg)
					Bolus (ml)	Continuous (ml/min)		
I	50	150	177–815	Off	10	Off	Off	–
II	25	75	815–780	1.5	–	Off	Off	–
III	85	255	780–670	1.8	–	1.2[a]	0.37	711
IV	10	30	670–680	1.8	–	Off	Off	–
V	23	69	680–747	1.8	–	Off	0.30	161
VI	11	33	747–769	1.8	–	Off	0.77	198
VII	Collection of all 30 RITP fractions after 204 min							124[b]

[a] Continuous feed was terminated after 70 min.
[b] From fractions 23 to 26.

rate of 0.37 ml/min. The BSA content in the bleed solution was 22.96 mg/ml, a value which is lower than that obtained previously after batch operation [5]. Under these conditions the throughput of BSA was calculated to be 510 mg/h and the amount recovered per unit charge spent was 2.79 mg. During this stage (and all subsequent stages with the bleed solution), the counterflow rate was increased to maintain the immobilized front.

After the termination of the continuous sample feed the system was stabilized for 10 min (stage IV) before further withdrawal of BSA (stages V and VI). The final processing fluid was fractionated as described for batch processes (stage VII). Fig. 2 shows the final absorbance, pH and conductivity distributions which were determined on the collected fractions. The protein content at this time was insufficient for the establishment of a plateau zone. The peak concentration (channel 24) was 14.9 mg/ml. The total recovery of BSA (stages I to VII) was 79% and the total charge spent was 612 C.

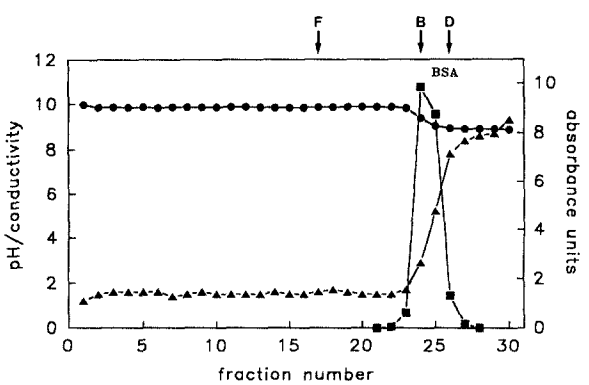

Fig. 2. RITP data after processing of 1.5 g of the BSA product with a total bleed of 1.07 g of BSA. Absorbance (280 nm, ■), pH (●) and conductivity (mS/cm, ▲) were measured on the collected fractions. D, B and F mark the positions of the boundary detector, bleed of purified product and sample feed, respectively.

Purification of LYSO and OVA by continuous cationic RITP

For all cationic RITP experiments a leader composed of 0.01 *M* potassium acetate and acetic acid (pH_L = 4.75) was used. The cathodic electrode compartment contained a ten-fold higher concentration of the same electrolyte, whereas 0.1 *M* acetic acid (terminator) was in the anodic electrode compartment. A commercial OVA product containing LYSO and CAL as impurities was used as the sample. Previous investigations by analytical ITP and RITP showed that GABA can be used successfully as a spacer between OVA and its two major proteinaceous impurities and tetrapropylammonium (TPA) to separate CAL and LYSO [6].

For the purification of LYSO, the best RITP results were obtained with TPA added to the anodic

TABLE II

EXPERIMENTAL STAGES FOR PURIFICATION OF LYSO FROM 2.1 g OF A COMMERCIAL OVA PRODUCT BY CONTINUOUS CATIONIC RITP

The time represents the time interval of a stage, the charge is the total charge spent during a stage, the voltage is given as the initial and end voltage of a stage, the counterflow values represent average flow-rates and the yield is the total amount of LYSO recovered during a stage.

Stage	Time (min)	Voltage (V)	Charge (C)	Counter-flow (ml/min)	Sample feed		Bleed rate (ml/min)	Yield of LYSO (mg)
					Bolus (ml)	Continuous (ml/min)		
I	53	144–342	159	Off	10	Off	Off	–
II	45	342–382	135	1.8	–	Off	Off	–
III	79	382–350	237	1.8	–	1.4	Off	–
IV	15	350–354	45	1.8	–	1.4	0.47	9.1
V	14	354–343	42	1.8	–	1.4	Off	–
VI	15	343–366	45	1.8	–	1.4[a]	0.27	4.9
VII	35	366–381	105	1.8	–	Off	Off	–
VIII	40	381–384	120	1.8	–	Off	0.23	13.8
IX	15	384–386	45	1.8	–	Off	Off	–
X	Collection of all 30 RITP fractions after 311 min							15.3[b]

[a] Terminates 3 min earlier.
[b] Fractions 22 to 25.

electrode solution [6]. This configuration allowed the isolation and purification of LYSO alone. During this experiment, OVA and CAL were first pushed by electromigration towards the membrane near the sample inlet and then swept out during the counterflow operation. For continuous operation this same configuration with 50 m*M* TPAB in the anolyte was used. First 715 mg of the OVA product were processed for 53 min (stage I of Table II) and counterflow was activated for 45 min to stabilize the system at a power level of about 20 W (stage II). Then continuous sample infusion and the withdrawal of purified LYSO could be begun in the same way as described for continuous anionic RITP of BSA (Fig. 1A). Computer simulation was used to obtain an estimated composition of the adjusted terminator of 4.34 m*M* TPA and 5.1 m*M* acetic acid (pH 4.37). A feed solution of 1.4 g OVA product dissolved in 200 ml of this buffer was prepared. The infusion rate was 1.4 ml/min. First LYSO was withdrawn at a rate of 0.47 ml/min with a concentration of 1.31 mg/ml (stage IV). The throughput and amount recovered per unit charge spent were 37 mg/h and 0.20 mg, respectively. The bleed solution was then stopped for 14 min (stage V) and continued at a rate of 0.27 ml/min (stage VI; the LYSO concentration and throughput were 1.19 mg/ml and 20 mg/h, respectively). After this no feed nor bleed occurred for 35 min (stage VII) before another withdrawal of LYSO at 0.23 ml/min (stage VIII; LYSO concentration and throughput were 1.52 mg/ml and 21 mg/h, respectively). Collection of the fluid in all recycling channels occurred after 311 min of operation and 933 C of charge spent. The analytical data obtained for the 30 fractions are shown in Fig. 3. It was interesting to find that the counterflow rate could be kept constant throughout this run.

The total amount of LYSO in fractions 22 to 25 was 15.3 mg. The total LYSO yield was 43.1 mg, which represents 2.1% of the applied amount of protein. For this run, the analytical data obtained by capillary ITP are shown in Fig. 4. Panel B shows data obtained after the injection of the commercial OVA product which was spiked with GABA and TPAB. The analysis of the bleed solution and fraction 22 (with the addition of the same two spacers) are shown in panels C and D, respectively. These data show that pure LYSO was collected.

In the two runs described here, the protein of interest established its ITP zone at the beginning of

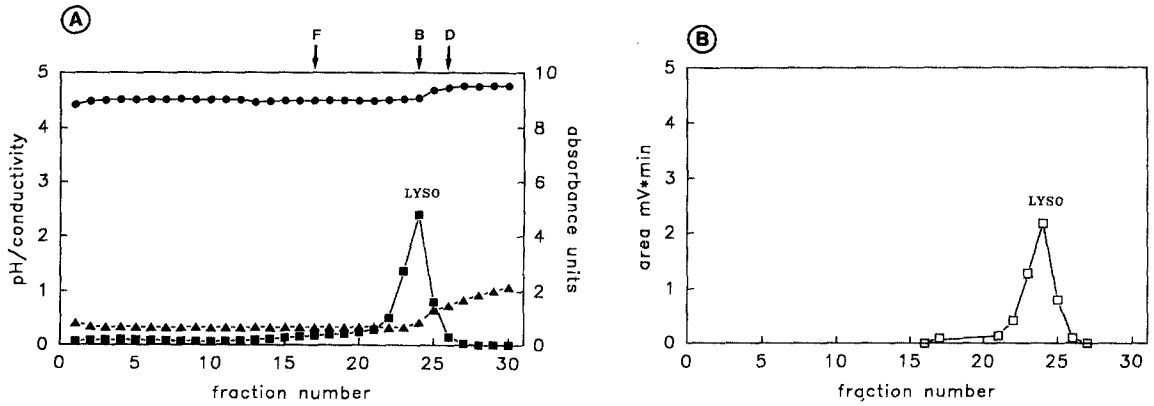

Fig. 3. RITP data after processing 2.1 g of the OVA product and bleeding off 27.8 mg of pure LYSO. Panel A shows absorbance, (280 nm, ■), pH (●) and conductivity (mS/cm, ▲) distributions measured on the collected fractions. Fractions containing CAL were filtered before analysis. Panel B shows the processed data of analytical capillary ITP runs on the Tachophor. Volumes of 1 μl of undiluted fractions and 1 μl of a spacer mixture of GABA and TPA were injected and analysed. Peak areas of the UV signals (277 nm) were determined with the PC integration pack. The peak concentration of LYSO (fraction 24, bleed channel) was 1.91 mg/ml. D, B and F mark the positions of boundary detector, bleed of purified product and sample feed, respectively.

the ITP sample stack. The third example presented in this paper consists of the continuous purification of OVA from the commercial OVA product. As this protein is the rear component in the cationic ITP stack in the potassium acetate–acetic acid system [5,6], the plumbing of the RITP set-up had to be changed (Fig. 1B). The UV monitor was inserted into recycling loop 6, which allowed the control of the rear edge of the OVA zone (Fig. 5). The sample was fed continuously into channel 17 (spacer zone) and purified OVA was withdrawn from channel 11. GABA was used as a spacer between OVA and

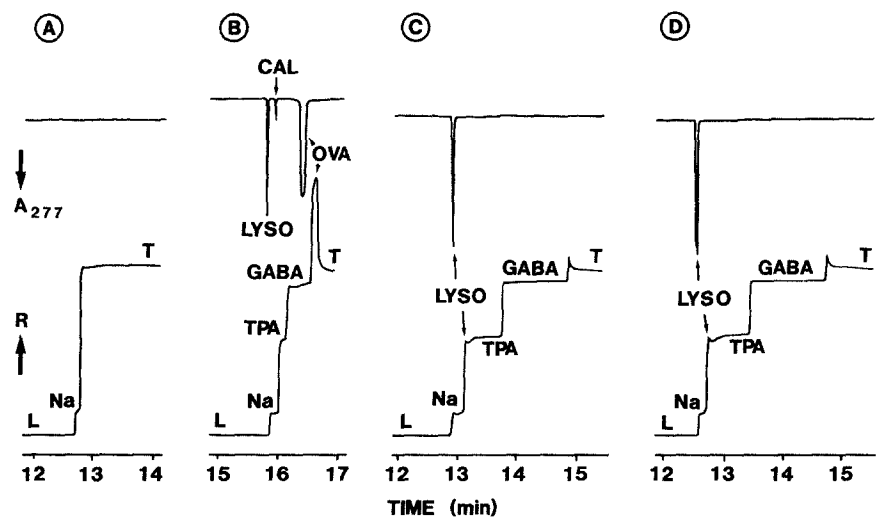

Fig. 4. Capillary ITP data for the purification of LYSO (Fig. 3, Table II) using the Tachophor analyser. Panel A shows the blank run, panel B the analysis of commercial OVA (feed), panel C the analysis of the bleed solution and panel D the analysis of fraction 24 after final fraction collection. Volumes of 1 μl of feed, bleed and the undiluted fraction and 1 μl of a spacer mixture of GABA and TPAB were injected and analysed. The lower and upper plots represent the conductivity (expressed as the increase in resistance *R*) and UV absorbance at 277 nm, respectively. L = Leader; T = terminator; Na = sodium as an impurity from electrolyte system.

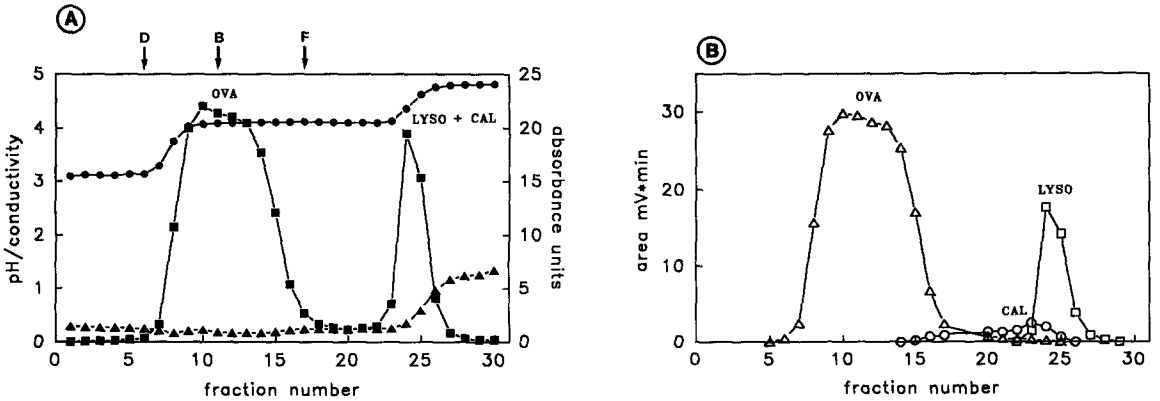

Fig. 5. RITP data after processing 2.1 g OVA and bleeding 659 mg of purified OVA. GABA was used as a spacer between OVA and the proteinaceous impurities. The OVA plateau concentration after final fraction collection was 35 mg/ml. For explanation of the data panels, refer to Fig. 3.

CAL/LYSO and the anolyte did not contain any additives. Initially, 706 mg of OVA and 32.7 mg of GABA were dissolved in 10 ml of 0.01 *M* leader and introduced through inlet 2. Cationic RITP was performed for 54 min (stage I of Table III) before 64 min of counterflow operation at a power level of about 24 W (stage II). Thereafter, the infusion of more sample occurred for 40 min at a rate of 0.6 ml/min (stage III). The feed solution was composed of 1.417 g of OVA product dissolved in 200 ml of 2.5 m*M* potassium acetate, which was adjusted to pH 4.08 (conductivity 28 mS/cm) with acetic acid. It is important to note that the feed solution did not contain any GABA, but had a similar conductivity to the established GABA spacer zone. OVA withdrawal was started at a rate of 0.12 ml/min (stage IV). The OVA concentration in the bleed solution was 22.2 mg/ml, a value which is again lower than the steady-state plateau concentration obtained with batch operation. Under these conditions a

TABLE III

EXPERIMENTAL STAGES FOR PURIFICATION OF 2.1 g OVA FROM A COMMERCIAL OVA PRODUCT BY CONTINUOUS CATIONIC RITP

The time represents the time interval of a stage, the charge is the total charge spent during a stage, the voltage is given as the initial and end voltage of a stage, the counterflow values represent average flow-rates and the yield is the total amount of OVA recovered during a stage.

Stage	Time (min)	Voltage (V)	Charge (C)	Counter-flow (ml/min)	Sample feed		Bleed rate (ml/min)	Yield of OVA (mg)
					Bolus (ml)	Continuous (ml/min)		
I	54	128–395	162	Off	10	Off	Off	–
II	64	395–471	192	1.8	–	Off	Off	–
III	40	471–593	120	1.0	–	0.6	Off	–
IV	132	593–638	396	1.3	–	0.6[a]	0.12	344
V	44	640–585	132	1.0	–	2.0[b]	0.36	315
VI	43	585–512	129	1.5	–	Off	Off	–
VII	Collection of all 30 RITP fractions after 377 min							800[c]

[a] Feed rate was changed to 2.0 ml/min after 111 min.
[b] Terminates 7 min earlier.
[c] Fractions 9 to 14.

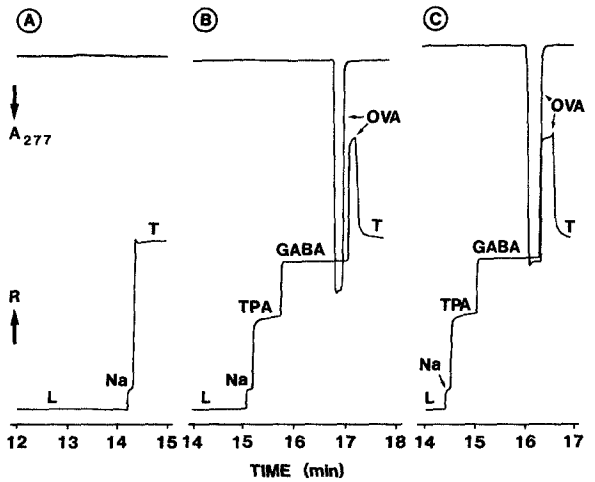

Fig. 6. Capillary ITP data of the OVA purification process (Fig. 5, Table III). Panel A shows the blank run, panel B the analysis of the bleed solution and panel C the analysis of fraction 11 after final fraction collection. Other conditions are as in Fig. 4.

yield of 156 mg/h and a recovery of 0.87 mg pure OVA per unit charge spent were obtained. A feed rate of 0.6 ml/min resulted in a protein infusion of about 250 mg/h. After 111 min of continuous operation the infusion rate was increased to 2.0 ml/min. The withdrawal rate was therefore increased to 0.36 ml/min (stage V). At this stage, the OVA concentration in the bleed solution was 19.7 mg/ml, which is equivalent to a yield of about 430 mg OVA per hour and a recovery of 2.39 mg OVA per unit charge spent. For the immobilization of the rear end of the OVA zone, the counterflow rate was readjusted for each stage.

Before the final fraction collection the system was run in the batch mode (stage VI). Collection of all recycling channels occurred after 377 min of operation and 1131 C of charge spent. A total amount of 1.46 g of pure OVA was recovered. This represents about 70% of the supplied amount of protein, a value which compares favourably with the OVA content of about 84% claimed by the supplier. Analytical data obtained for the 30 fractions are shown in Fig. 5 and the capillary ITP data are shown in Fig. 6. These analyses show that pure OVA was obtained and that, within the experimental time frame, the accumulation of CAL and LYSO did not disturb this process. An overload of these compounds is expected with longer run times, a situation which would require the continuous (or batch) removal of these proteins, *e.g.* from recycling loop 25. With a selected amount of TPAB in the original sample, and with the same instrumental set-up, it is anticipated that purified OVA and LYSO could be recovered simultaneously and continuously.

CONCLUSIONS

RITP is a free fluid electrophoretic approach for the fractionation of proteins which features a continuous and batch mode of operation. The former mode, for which the first experimental data are reported here, is more attractive than the latter, particularly for the incorporation of RITP as a downstream processing unit operation, *i.e.* as a purification method in an industrial multistep purification protocol. As is shown with the example of BSA, the processing rate in continuous RITP is comparable with that obtained in continuous flow ITP [4]. In the examples of BSA and LYSO, the compounds to be purified established their ITP zones at the front of the sample stack. This represents the simplest example of continuous RITP in which the feed is infused into the adjusted electrolyte behind the target zone. Most sample impurities are thereby quickly removed and swept out of the cell by counterflow. Continuous purification of a compound located at the rear of the isotachophoretic zone structure, as for the OVA example, requires a more complex plumbing with sample infusion in front of the target zone in addition to occasional or continuous removal of proteinaceous impurities which accumulate within the separation cell. For compounds which form their ITP zone at neither edge of the stack, the experimental configuration has to be adapted for each sample separately and continuous purification will always be incomplete because of the migration of some contaminants through the sample zone. In this instance a semi-continuous mode of operation in which sample infusion and the withdrawal of the purified product are alternated is proposed. Such a procedure also requires an occasional removal of accumulated proteinaceous impurities, but allows a more complete purification of the centre compounds. Splitting the protein matrix by feed and bleed recycling zone electrophoresis [8] followed by purification by continuous RITP as an

edge compound would be another, but two-stage, approach worth considering.

The investigated purification of LYSO (about 40 mg/h) and OVA (about 500 mg/h) from a commercial OVA sample show the capability of using RITP to continuously isolate a minor component (here approximately 3% of the protein content) and purify a major sample constituent, respectively. Without the optimization of the various running parameters, the purities of the products were determined to be equal and the achieved throughputs were higher than those obtained in batch mode operation [6]. Scaling-up of the procedure to even higher throughputs and full automation of the continuous process are currently being studied.

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REFERENCES

1 J. E. Sloan, W. Thormann, M. Bier, G. E. Twitty and R. Mosher, in M. J. Dunn (Editor), *Electrophoresis '86*, VCH, Weinheim, 1986, p. 696.
2 J. E. Sloan, W. Thormann, G. E. Twitty and M. Bier, *J. Chromatogr.*, 457 (1988) 137.
3 M. Bier, G. E. Twitty and J. E. Sloan, *J. Chromatogr.*, 470 (1989) 369.
4 W. Thormann, M. A. Firestone, J. E. Sloan, T. D. Long and R. A. Mosher, *Electrophoresis*, 11 (1990) 298.
5 J. Caslavska, P. Gebauer, A. Odermatt and W. Thormann, *J. Chromatogr.*, 545 (1991) 315.
6 J. Caslavska, P. Gebauer and W. Thormann, *J. Chromatogr.*, 585 (1991) 145.
7 W. Thormann and R. A. Mosher, *Electrophoresis*, 11 (1990) 292.
8 M. Bier, N. B. Egen, G. E. Twitty, R. A. Mosher and W. Thormann, in C. J. King and J. D. Navratil (Editors), *Chemical Separations, Vol. 1: Principles*, Litarvan Literature, Denver, 1986, p. 133.