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Purification of ovalbumin and lysozyme from a commercial product by recycling isotachophoresis

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ABSTRACT

The aim of this work was to test the suitability of using recycling isotachophoresis (RITP) for the purification of ovalbumin (OVA) and/or lysozyme (LYSO) from a commercial OVA product containing LYSO and conalbumin (CAL) as major proteinaceous impurities. The search for suitable electrolyte systems and spacers was carried out by capillary isotachophoresis. RITP was performed in a recycling free-flow focusing apparatus in the batch mode with immobilization of the advancing zone structure via a controlled counterflow. Typically 700 mg of the commercial product were processed within 2 h. Enhancement of the sample load was achieved by a feed of sample under counterflow control. The collected fractions were analysed separately for conductivity, pH and ultraviolet absorption, and selected fractions were characterized by analytical capillary electrophoretic methods. All three proteins could be separated and fractionated using suitable spacers. Depending on the chosen conditions either OVA or LYSO could be purified in amounts larger than milligrams per hour (OVA 300 mg/h; LYSO 10 mg/h). The instability of CAL in solution prevented its isolation in the investigated configurations.

INTRODUCTION

Most investigations involving preparative isotachophoresis (ITP) use solid support media (for overviews, see Holloway and Battersby [1] and Sloan *et al.* [2]). Several other papers indicate a renaissance of using free-fluid preparative ITP as a purification method for proteins. Continuous-flow ITP [3–5], recycling ITP (RITP) [2,5,6] and screen-segmented column ITP [6] are currently being investigated; these methods provide high resolution coupled with high protein concentration, large throughput and control over fractionation pH, as well as the potential for automation. The purification of monoclonal antibodies from mouse ascites fluid and tissue culture media [7] and the fractionation of apolipoprotein B-containing lipoproteins from fasting and postprandial sera derived from normolipedaemic individuals [8] are two recent applications using continuous-flow ITP. This laboratory is in the process of exploring RITP as a free-fluid approach for the ITP determination of proteins on the gram scale [2,5,6]. 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 cell is of the order of 1 s 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.

Egg proteins are important constituents of the human diet and have been the subject of many investigation. The isolation of egg proteins, such as ovalbumin (OVA), is typically performed through repetitive crystallization, salt removal and lyophilization [9]. One paper reports the fractionation of egg white proteins by recycling isoelectric focusing [10]. A commercial OVA was found to contain major proteinaceous impurites which, in a cationic ITP configuration around a leader pH of 4.75, migrate

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in front of OVA [6]. It was demonstrated that using γ-amino-*n*-butyric acid (GABA) as a spacer, OVA could be purified by RITP and determination by capillary ITP revealed that the major OVA fractions were essentially free of these impurities. This example constitutes an interesting model system for an in-depth evaluation of RITP. This sample was therefore further investigated and this paper reports the removal, separation and identification of the two major proteinaceous impurities, lysozyme (LYSO) and conalbumin (CAL), as well as the purification of LYSO compared to that of OVA.

EXPERIMENTAL

Chemicals

All chemicals used were of research-grade purity.

Tris(hydroxymethyl)aminomethane (TRIS), GABA and hydroxypropylmethylcellulose (HPMC, No. 7509) were from Sigma (St. Louis, MO, USA). OVA from chicken egg (5 × crystallized, Lot No. 11840/D8), LYSO from chicken egg white, CAL and tetrapropylammonium bromide (TPAB) were from Serva (Heidelberg, Germany). Tetrabutylammonium bromide (TBAB) was purchased from Fluka (Buchs, Switzerland) and creatinine (CREAT), potassium acetate and acetic acid were from Merck (Darmstadt, Germany).

RITP

The RITP instrument used in this work is the same as described previously [6]. Briefly, it consists of a recycling free-flow focusing apparatus (Model RF3, Protein Technologies, Tucson, AZ, USA; distributor, Rainin Instrument, Woburn, MA, USA) with modification 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 monitored to be about 13°C (cooling bath 2–5°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 250 ml (RF3 standard 60 ml) were used and filled with buffers of ten-fold higher concentration than those used within the separation cell. Unless otherwise stated, 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 2138 Uvicord S spectrometer (Pharmacia LKB Biotechnology, Uppsala, Sweden) with a 277-nm filter. The detector was inserted into the recycling loop of channel 26 (near the leading electrolyte chamber) The counterflow inlet and outlet were placed into 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 bubble trap, and was either regulated manually with registration of the output signal from the boundary detector on a strip-chart recorder or automatically using a REX-G9 high-performance digital controller (RKC Instrument, Tokyo, Japan).

All experiments were performed in the batch mode. The separation cell was filled with the leading electrolyte. The multichannel peristaltic pump was set to a pumping rate of 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 the power was applied at a constant current of 50 mA. When the absorbance changed in the monitoring loop, the counterflow was activated to maintain a constant absorbance level. The ITP zone structure was thereby immobilized. Typically the counterflow pumping rate did not exceed 3.0 ml/min.

Analysis of collected fractions

For pH measurements a Model 720 pH meter and a ROSS Model 8103 SC 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–visible Lambda 15 spectrophotometer (Perkin-Elmer, Überlingen, Germany). In some instances the fractions containing the protein zones

had to be diluted ten times and for presentation of the data the absorbance values were multiplied by ten. Some CAL-containing fractions with high turbidity had to be filtered prior to analysis.

Selected fractions and model mixtures were analysed by capillary ITP using a Tachophor 2127 analyser (LKB, Bromma, Sweden). This instrument was equipped with a 28 cm × 0.5 mm I.D. PTFE capillary and 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 registered with a two-channel strip-chart recorder and/or with a data acquisition system comprising a PC integration pack (version 2.50, Kontron Instruments, Zürich, Switzerland), together with a Mandax AT 286 computer system. This integration pack features two channels for data acquisition, automatic range switching and a dynamic sampling rate, allowing sampling every 10 ms for quickly changing signals. The leader employed was composed of 0.01 *M* potassium acetate and acetic acid (pH_L = 4.75). The terminator was 0.01 *M* acetic acid.

Similar analyses were performed in a laboratory-made capillary electrophoretic analyser described in detail previously [11]. Briefly it features a 90 cm × 75 μm I.D. fused-silica capillary (Product TSP/075/375, Polymicro Technologies, Phoenix, AZ, USA), together with a fast-scanning multiwavelength Model UVIS 206 PHD detector and an on-column capillary detector 9550-0155 cell (both of Linear Instruments, Reno, NV, USA) placed towards the capillary end. The effective separation distance was 70 cm. Sample application occurred manually via gravity through lifting the anodic capillary end, dipped into the sample vial, by 34 cm for a specified time interval. Multiwavelength data were read, evaluated and stored using a Mandax AT 286 computer system and running the 206 detector software package version 2.0 (Linear Instruments) with Windows 286 version 2.1 (Microsoft, Redmont, WA, USA). Throughout this work the 206 detector was used in the high-speed polychrome mode by scanning from 195 to 320 nm at 5-nm intervals (26 wavelengths). The same electrolytes as with the Tachophor were used, except the leader contained 0.3% HPMC. All runs were performed with a constant voltage of 20 kV. During an experiment, the current decreased from 12 μA (initial) to about 4 μA (time of detection). The details of protein analyses with this instrument are described elsewhere [12].

RESULTS AND DISCUSSION

For all RITP experiments, a cationic ITP system with 0.01 *M* potassium acetate and acetic acid (pH_L = 4.75) as leader 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 well as separate commercial LYSO and CAL samples, were used. Previous investigations by analytical ITP and RITP showed that GABA can successfully be used as a spacer between OVA and its two major proteinaceous impurities [6]. RITP fractionation data of a commercial sample of OVA (710 mg of OVA from Serva 11840/D8 with 15 mg GABA) into pure OVA and CAL–LYSO is presented in Fig. 1A. In this experiment, immobilization of the advancing zone pattern was obtained via automated regulation of the counterflow employing a digital controller. The OVA plateau concentration (fraction 16) was 22 mg/ml and the typical processing throughput was calculated as 300 mg/h. Comparing these results with previously reported data [6] reveals that the automated regulation provides broader zone boundaries than with a manually controlled counterflow. The protein distribution in the collected fractions was analysed by capillary ITP in the Tachophor after spiking the sample with GABA and TRIS. The areas of the UV absorption peaks, determined by manual integration, are shown in Fig. 1B. These data show that the separation of OVA and its major proteinaceous impurities was successful and that LYSO migrates ahead of CAL. The goals of the following experiments were the separation of LYSO and CAL and the purification of LYSO.

Investigations using capillary ITP revealed that CAL and LYSO separate well in the given electrolyte system using different spacers. Typical pherograms obtained on the Tachophor are presented in Fig. 2. The ITP zone formation of the two proteins without spacer is shown in Fig. 2A. LYSO migrates ahead of CAL, their separation being indicated by a sharp UV signal spike which marks the location of the common interface. TRIS (Fig. 2B) was found to

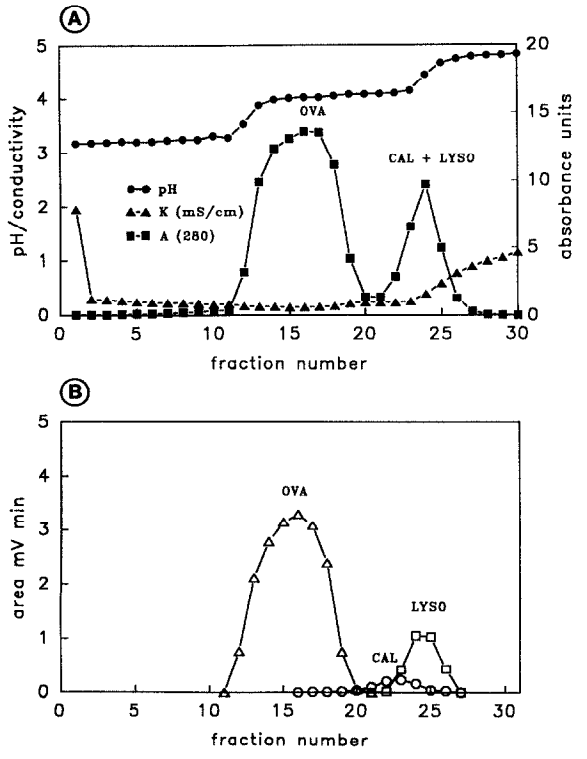

Fig. 1. RITP data after processing 710 mg of OVA and 15 mg of GABA. The voltages across the cell assembly at the beginning of counterflow (60 min) and at collection time (133 min) were 518 and 558 V, respectively. The total charge applied was 399 C. (A) Absorbance, pH and conductivity distributions measured on the collected fractions; (B) processed data of analytical capillary ITP runs on the Tachophor. Volumes of 1 μl of the undiluted fractions and 1 μl of a spacer mixture of GABA and TRIS were injected and analysed. For each fraction and protein the peak area of the UV signal (277 nm) was plotted.

separate the two proteins. Its net mobility, however, is too fast for proper separation with LYSO. Therefore no resolution was obtained using this spacer in RITP (data not shown). According to the analytical data (Fig. 2C), CREAT should be a suitable spacer for the separation of LYSO and CAL. However, the RITP data depicted in Fig. 3 show that this was not the case. In the first experiment (Fig. 3A) 7.54 mg of LYSO, 3.00 mg of CAL and 31.33 mg of CREAT were processed for 47 min and fractionated without counter flow. Significant mixing of the two proteins within the spacer zone was observed and no steady state was reached. The data from a similar experiment but with the application of counter flow for 194 min are shown in Fig. 3B. Although the operation of counter flow improved the separation, some mixing between LYSO and CREAT was still present. Therefore, CREAT in that system cannot be used for an efficient RITP purification of LYSO. Furthermore, tetrabutylammonium (TBA, Fig. 2E) was too slow for the proper separation of the two proteins of interest. In RITP experiments, LYSO was strongly enriched at the front boundary of the spacer, but CAL was distributed across the entire TBA zone.

The best RITP results were obtained with tetrapropylammonium (TPA) as spacer, a configuration which also provided good analytical data (Fig. 2D). RITP data showing the removal of OVA and the separation of LYSO and CAL with TPA are presented in Fig. 4. The sample was composed of 740 mg OVA and 64 mg TPAB, processed for 60 min without counterflow followed by 206 min of manually controlled counterflow. GABA (0.05 *M*) was added to the anodic electrode solution and therefore acted as a terminator in the separation cell. This resulted in the accumulation of OVA at the membrane which separates the anodic electrode compartment from the cell (recycling channel 1) and its complete removal from the system during counterflow operation. Neither protein shows a plateau concentration at the given sample load. The peak concentration of LYSO (fraction 23) was 2.25 mg/ml. A similar example but with TPAB (0.05 *M*) instead of GABA as the additive to the anodic electrode solution is presented in Fig. 5. Here 710 mg of the OVA product was processed and counterflow was activated for 213 min. This configuration allowed the isolation and purification of LYSO alone. During that experiment, both OVA and CAL were first pushed by electromigration towards the membrane near the sample inlet and then swept out during counterflow operation. The total amount of LYSO in fractions 18–26 was determined to be 21 mg and its processing rate 4.6 mg/h. For that run analytical data obtained in both capillary instruments are shown in Figs. 6 and 7. Panels B of the two figures depict data obtained after injection of the commercial OVA product, which was spiked with GABA and TPAB. It was interesting that the small amount of CAL in the OVA sample could not be detected with a fused-silica capillary (Fig. 6B), but could be readily monitored in the PTFE capil-

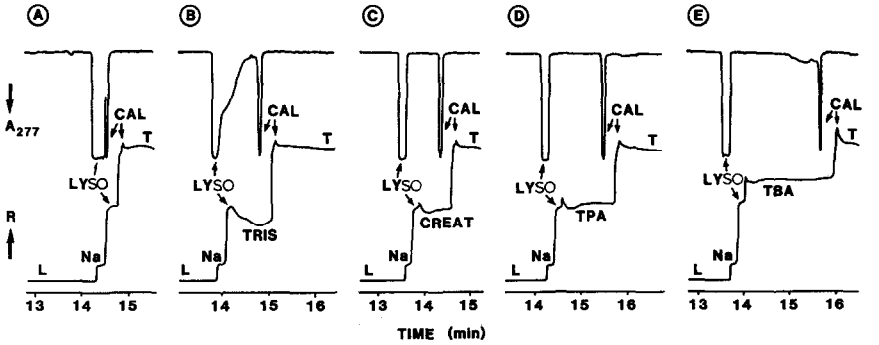

Fig. 2. Capillary ITP data obtained with model mixtures of commercial LYSO and CAL on the Tachophor. (A) Data without a low-molecular-mass spacer; (B–E) corresponding data with TRIS, CREAT, TPA and TBA as spacers, respectively. The lower and upper graphs represent the conductivity (expressed as a increase in resistance *R*) and UV absorbance at 277 nm, respectively. L = leader; T = terminator; Na = sodium impurity from electrolyte system.

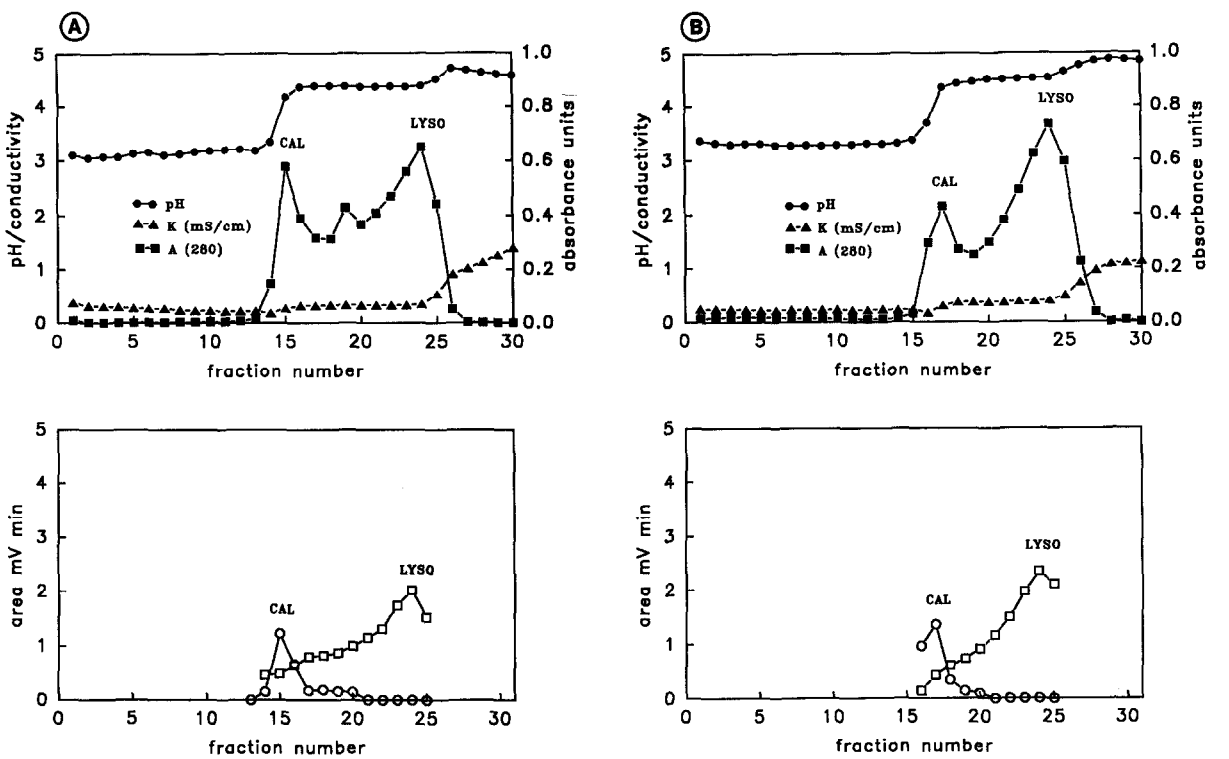

Fig. 3. (A) RITP data of LYSO (7.54 mg), CREAT (31.33 mg) and CAL (3.00 mg) after processing for 47 min (total charge 144 C) without counterflow; (B) Similar data with 7.00 mg LYSO, 31.87 mg CREAT and 3.00 mg CAL after running for 47 min without and 194 min with a manually controlled counterflow (total processing charge 737 C). The upper graphs depict absorbance, pH and conductivity distributrions measured on the collected fractions. The lower graphs show processed data of analytical capillary ITP runs on the Tachophor. A 7-μl volume of undiluted fractions spiked with additional CREAT was injected and analysed. Peak areas were determined with the PC pack.

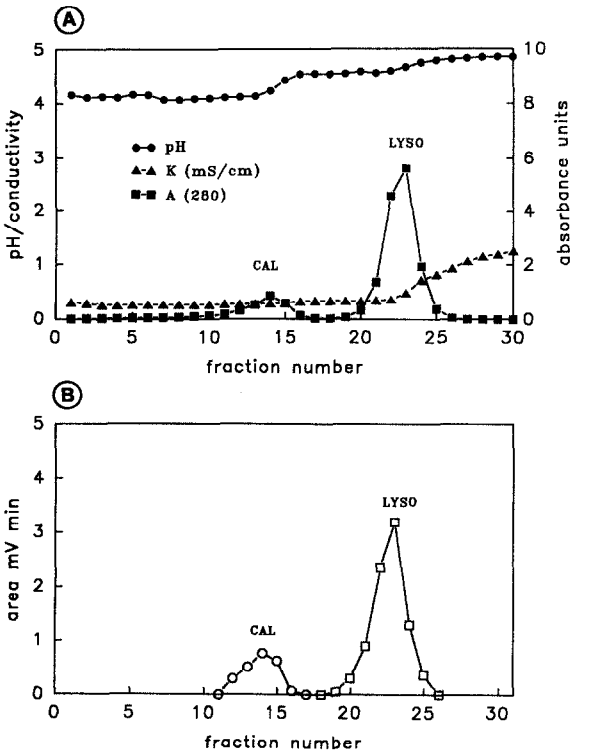

Fig. 4. RITP data after processing of 740 mg of OVA and 64 mg of TPAB with 0.05 *M* GABA in the terminating electrolyte. The total voltages at the beginning of counterflow and collection time were 354 and 404 V, respectivley, and the total processing charge was 801 C. (A) Absorbance, pH and conductivity distributions measured on the collected fractions. The CAL-containing fractions were filtered prior to analysis. (B) Processed data of analytical capillary ITP runs on the Tachophor. A 1-μl volume of undiluted fractions and 1 μl of a spacer mixture of GABA and TPA were injected and analysed. Peak areas were determined with the PC pack.

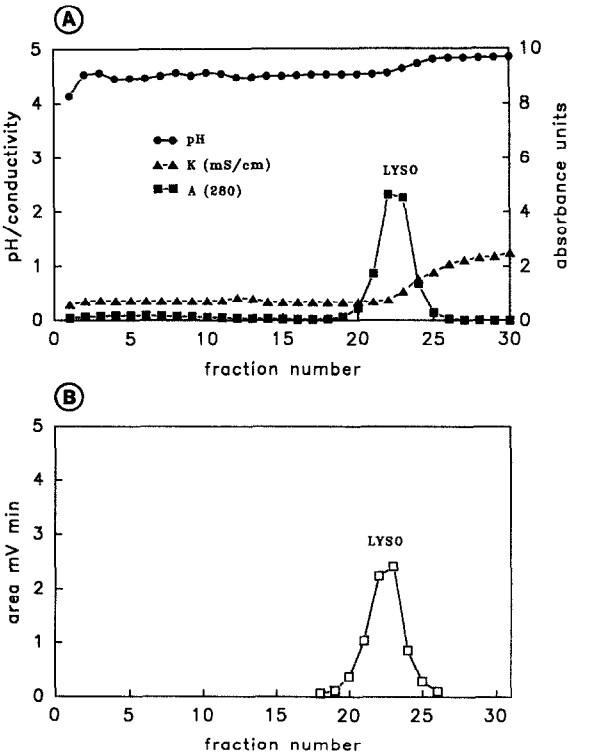

Fig. 5. RITP data after processing of 710 mg of OVA with a mixture of 0.05 *M* TPAB and 0.1 *M* acetic acid as terminator. The total voltages at the beginning of counterflow and collection time were 304 and 324 V, respectively, and the total charge was 819 C. The peak concentration of LYSO (fraction 22) was 1.86 mg/ml. Other conditions are the same as in Fig. 4.

lary (Fig. 7B) (see Gebauer and Thormann [12] for further explanations). The analysis of fraction 22 (with the addition of the same two spacers) is shown in panels C of the two figures. The isolated LYSO is fairly pure as determined by spectral analysis of the protein peak (Fig. 6).

Owing to a solubility restriction of the crude OVA product and a limitation of the applicable sample volume to about 10 ml at the beginning of a RITP run, not much more than 700 mg of the crude OVA product could be loaded onto the apparatus in one step. For this reason, other possibilities of sample introduction were investigated. Fig. 8 shows a possible solution to increasing the sample load, *i.e.* using a continuous sample feed mode of operation during part of the experiment. In other respects, the run was similar to that shown in Fig. 5. First RITP was performed with 710 mg of crude OVA as described earlier and with applied counterflow for 60 min. Thereafter additional sample (710 mg of OVA dissolved in 110 ml of leader) was infused (1.91 ml/min) through counterflow action. This was followed by counterflow of pure leader for 78 min prior to sample collection. Analysis of fractions 16–27 revealed a yield of 46 mg of LYSO and the processsing rate was 10.6 mg/h. Compared with the run depicted in Fig. 5 substantially more LYSO could be purified with the same amount of charge passed, but, at collection time, some residual OVA

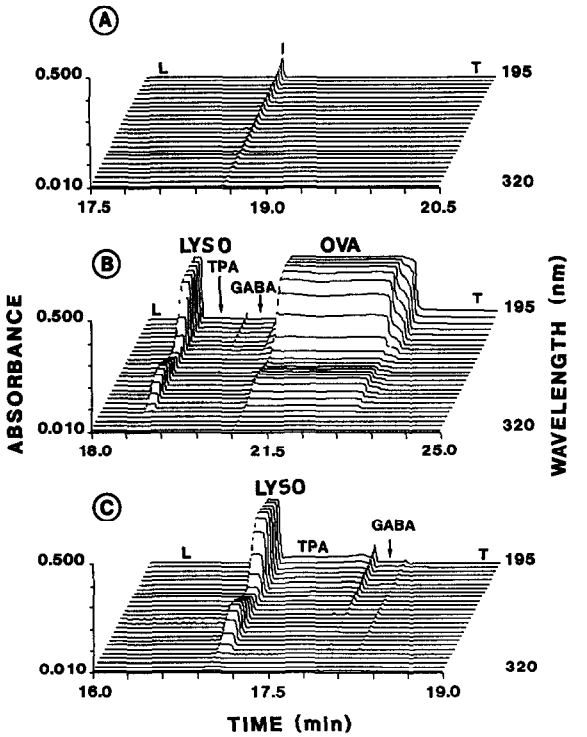

Fig. 6. Capillary ITP data of the purification depicted in Fig. 5 using a 75 μm I.D. fused-silica capillary and a scanning UV absorbance detector towards the capillary end. (A) Blank; (B) analysis of commercial OVA spiked with GABA and TPAB; (C) analysis of fraction 22 together with the two spacers. L = leader; I = impurity; T = terminator.

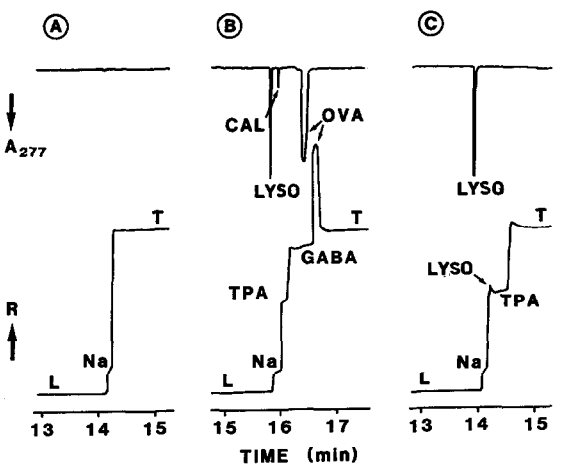

Fig. 7. Capillary ITP data of the purification depicted in Fig. 5 using the Tachophor analyser. Other conditions are the same as in Fig. 6.

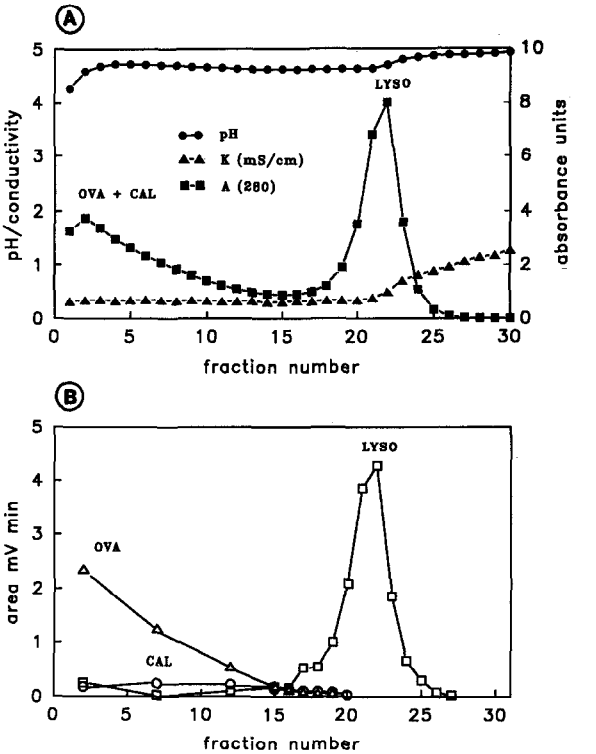

Fig. 8. RITP data of a run with first 710 mg of OVA (60 min until activation of a 60 min counterflow), then counterflow infusion of another 710 mg of OVA dissolved in the leader for 63 min, followed by a final counterflow operation for 78 min. The voltages across the cell at the beginning of counterflow, the beginning and end of counterflow sample infusion, and at collection time were 317, 327, 296 and 302 V, respectively. The total processing charge was 783 C. The peak concentration of LYSO (fraction 22) was 3.2 mg/ml. Other conditions are the same as in Fig. 5.

and CAL were still present in the processing solution.

CONCLUSIONS

RITP is an attractive free-fluid electrophoretic approach for the fractionation of proteins. Depending on selected spacers and working conditions both minor and major proteins can be separated and purified from the same crude sample. The data on the purification of LYSO (up to 10 mg/h) from a commercial OVA sample demonstrate the capability of using RITP to isolate a minor component (here approximately 3% of the protein content),

whereas the RITP purification of OVA itself (300 mg/h) represents an attractive polishing step for that protein. Scale-up to higher throughputs and a continuous mode of operation are the subjects of forthcoming investigations.

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