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Isotachophoresis of proteins in uncoated open-tubular fused-silica capillaries with a simple approach for column conditioning

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ABSTRACT

The isotachophoretic determination of proteins in uncoated open-tubular fused-silica capillaries of 50 and 75 μm I.C. with on-column multi-wavelength detection is reported. Small amounts of hydroxypropylmethylcellulose added to the leader provide an efficient method of dynamic column conditioning which allows the high-resolution isotachophoretic determination of most proteins to be performed in the presence of an electro-osmotic flow. Different approaches for cationic and anionic analyses are discussed and illustrated with selected examples.

INTRODUCTION

For more than 20 years, most isotachophoretic (ITP) analyses were performed in narrow-bore plastic tubes of 200–500 μm I.D. or separation channels of rectangular cross-section and with minimized electro-osmosis [1,2]. Modern capillary-type electrophoretic analysers feature fused-silica capillaries of 25–100 μm I.D. which can exhibit strong electro-osmotic flows. Work by Smith and co-workers [3–5], Hjertén and co-workers [6,7], Thormann and co-workers [8–11] and Beckers *et al.* [12] has shown that anionic and cationic ITP analyses can be performed in both untreated and coated open-tubular fused-silica capillaries, *i.e.* in the presence of and with minimized electro-osmosis, respectively. With these columns, most of the solutes investigated so far encompass low-molecular-mass compounds which show minimal interaction with the column walls.

In the ITP analysis of proteins, zones of high concentration (typically of the order of 10–20 mg/ml [8]) are formed. The proteins are vulnerable to interactions with the column walls. Not surprisingly, experiments with proteins in untreated fused-silica capillaries could not be reproduced, whereas much improved data were ob-

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tained in capillaries coated with linear polyacrylamide [6–8] or with a bonded and cross-linked film of DB-17 [4,5]. The best capillary ITP results for proteins were obtained with PTFE capillaries [13]. This paper reports a simple approach for capillary conditioning which allows cationic and anionic analyses of proteins to be performed in untreated open-tubular fused-silica capillaries. ITP analysis with multi-wavelength detection is shown to represent a useful tool for the characterization of protein samples.

EXPERIMENTAL

Chemicals

All chemicals used were of research grade purity. Ribonuclease A (RNase) from bovine pancreas, carbonic anhydrase (CA) from bovine erythrocytes, γ-amino-*n*-butyric acid (GABA), 2-amino-2-methyl-1,3-propanediol (ammediol), β-alanine and hydroxypropylmethylcellulose (HPMC, No. 7509) were from Sigma (St. Louis, MO, USA). Ovalbumin (OVA) from chicken egg (5 × crystallized, Lot No. 11840 D8), lysozyme (LYSO) from chicken egg white and conalbumin (CAL) were from Serva (Heidelberg, Germany). Bovine serum albumin (BSA) was purchased from Fluka (Buchs, Switzerland) and creatinine (CREAT), potassium acetate, formic acid and acetic acid were from Merck (Darmstadt, Germany). Ampholine (pH 3.5–10) was obtained from Pharmacia-LKB (Bromma, Sweden).

Electrolyte systems and sample preparation

For all the experiments presented here two electrolyte systems were used, a cationic system 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, and an anionic system composed of 10 m*M* formic acid titrated with ammediol to pH_L 9.1 (leader) and 10 m*M* β-alanine–ammediol of pH 9.5 (terminator). In most instances 0.3% HPMC was added to the leaders. Sample components were dissolved in the leader without the addition of HPMC. Protein concentrations of 10–30 mg/ml were used.

Instrumentation and running conditions

The laboratory-instrument used in this work has been described previously [10]. It featured either a 50 or 75 μm I.D. fused-silica capillary of about 90 cm length (Polymicro Technologies, Phoenix, AZ, USA), together with a fast-scanning multi-wavelength detector (Model UVIS 206 PHD) and a capillary detector cell No. 9550-0155 (both from Linear Instruments, Reno, NV, USA). The effective separation distance was about 70 cm. Two 50-ml plastic bottles served as electrode vessels and a vacuum pump (Model VacTorr 150, CGA/Precision Scientific, Chicago, IL, USA) was used to rinse the capillary. Current was applied at a constant voltage (20 kV) with a Model HCN 14-20000 power supply (FUG Elektronik, Rosenheim, Germany). Sample application occurred manually via gravity through lifting the capillary end, dipped into the sample vial, some 34 cm for a specified time interval. Multi-wavelength 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 employed in the high-speed polychrome mode by scanning from

195 to 320 nm at 5-nm intervals (26 wavelengths). New capillaries were first rinsed with 1 *M* sodium hydroxide (20 min) and then 0.1 *M* sodium hydroxide (10 min). Before each run the capillary was cleaned with 0.1 *M* sodium hydroxide (10 min) followed by buffer insertion for at least 10 min.

RESULTS AND DISCUSSION

In the cationic mode, the cathode was on the detector side and the entire capillary was filled with leader prior to sample analysis. In this configuration both electro-osmotic and electrophoretic sample displacement occurred towards the detector. Experiments without the addition of a polymer to the solution were performed first. It was interesting to find that with the 50 μm I.D. capillary none of the six tested proteins could be monitored within 30 min after the application of a constant voltage of 20 kV. This is in contrast to the behaviour of low-molecular-mass compounds, which form sharp ITP zones and could be detected within a few minutes [9]. With a 75 μm I.D. capillary, the situation was improved. Some proteins, such as LYSO and CA, did reach the detector, but the sample zones were poorly defined and the elution times were unexpectedly high compared to low-molecular-mass constituents [10]; others, such as CAL and OVA, could not be detected within 40 min of current application. It is presumed that the proteins are adsorbed onto the capillary walls, thereby forming a coating which changes the surface charge and thus the electro-osmotic flow (reduction or even reversal of the net sample flux).

The addition of HPMC to the leader was found to allow the formation and migration of well developed cationic ITP zones of many proteins. The cationic ITP behavior of CA in the 75-μm capillary is shown in Fig. 1. Panel A depicts the three-

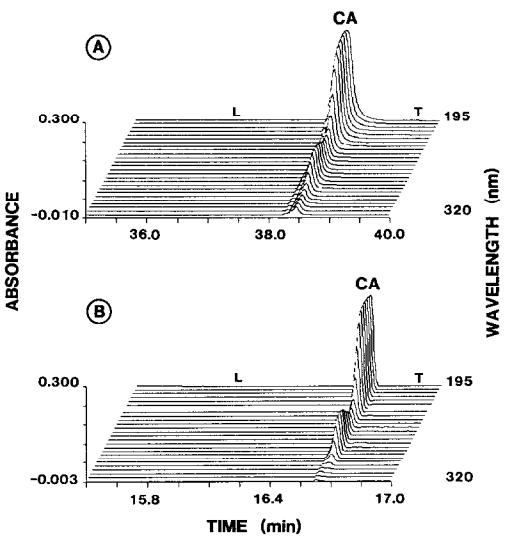

Fig. 1. Three-dimensional isotachopherograms of the cationic analysis of CA in a 75 μm I.D. fused-silica capillary (A) without and (B) with HPMC in the leader. Sample application occurred for 20 and 60 s, respectively, with about 10 mg/ml CA dissolved in the leader. The initial and final currents (at detection time) were 10 and 3, and 11 and 4 μA, respectively. L and T refer to leader and terminator, respectively.

dimensional data plot of CA without the addition of HPMC. A rather broad zone without sharp edges eluting after about 38 min of current application was monitored. With 0.3% HPMC in the leader, this zone is much better defined and elution occurred within 15–18 min (panel B), a time interval which is almost twice a high than that observed with low-molecular-mass compounds an in the absence of HPMC [10]. As the current densities are equal in the two approaches, HPMC obviously lowers the electro-osmotic flow. This effect was previously noted for 220 μm I.D. capillaries [14].

Fig. 2 shows three-dimensional electropherograms of a blank (panel A), of a model mixture composed of LYSO, CAL and OVA with the spacers CREAT and GABA (panel B) and of a commercial OVA sample which was spiked with two low-molecular-mass spacers (panel C). All these experiments were performed with 0.3% HPMC in the leader and sufficient sample was introduced to allow the formation of plateau zones in panels B and C. Sharp boundaries are formed which are marked by absorbance changes. Thus distinction between the zones is simple, even for the blank where small amounts of impurities mark the leader–terminator transition (panel A). With the model mixture (panel B), conditions for the ITP analysis of the three proteins were elucidated. LYSO and OVA were found to establish proper ITP zones, the lengths of which linearly increased with the addition of more sample.

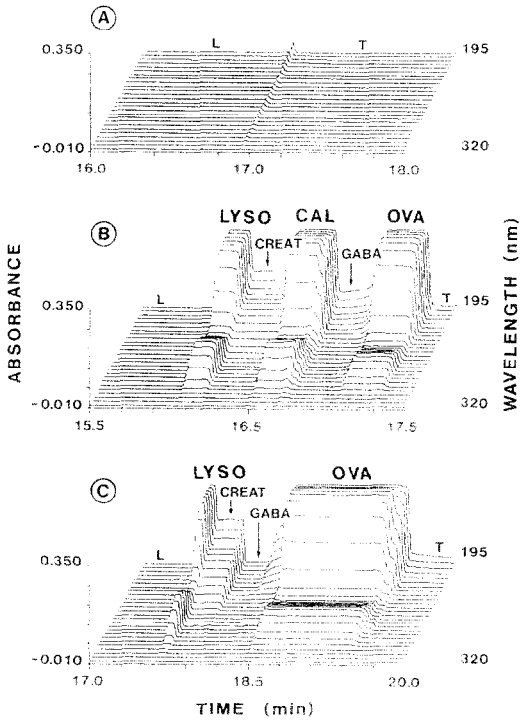

Fig. 2. Three-dimensional isotachopherograms for cationic analysis of (A) blank, (B) LYSO, CREAT, CAL, GABA and OVA and (C) OVA spiked with CREAT and GABA in a 75 μm I.D. capillary. The leader of all three experiments contained HPMC. The initial and final currents (at detection time) for the blank run were 12 and 2 μA, respectively.

CAL, however, behaved differently. Despite the presence of HPMC a loss of part of the protein was observed. This is particularly seen with the data shown in panel C. The commercial OVA product used in this study contained two major proteinaceous impurities, LYSO and CAL [15]. ITP analysis in a fused-silica capillary (as presented here) revealed the presence of LYSO only, whereas the same analysis in a PTFE capillary showed both proteins (date not shown; similar data are given in Caslavska *et al.* [15]).

With spacer compounds the purity of a protein can be assessed. As is shown in panel C of Fig. 2, the addition of a couple of well characterized, low-molecular-mass spacers can separate proteins by forming their own ITP zones. With sample detection over a range of wavelengths in one experiment, both protein and spacer zones are fully characterized. The identification of compounds via comparison of spectra, as is shown for OVA, LYSO and CREAT in Fig. 3, is possible. The presented spectra represent time slices extracted from the data of Fig. 2B. The described technique can also be used for the purity control or fractionation of proteins in the presence of a large number of spacer molecules, such as the carrier ampholytes manufactured for isoelectric focusing [13]. The three-dimensional electropherogram of RNase depicted in panel A of Fig. 4 shows that this protein forms an ITP zone with substantial impurites at its rear edge. These impurities are removed from RNase through the addition of carrier ampholytes to the sample (panel C). This analysis was only successful in the presence of HPMC and compared well with data obtained in a PTFE capillary (data not shown).

In the anionic mode protein ITP is predominantly executed at high pH. With the leader in the electrode vessel on the detector side, as well as initially throughout the entire capillary, and the anode on the detector side, no results are provided within 30 min of power application. Therefore the reversed buffer configuration reported previously for low-molecular-mass substances [9] was used. Initially, the capillary and the electrode vessel near the detector contained the terminator, whereas the leader was in the electrode compartment on the sample inlet side. The cathode was on the detector side. In this configuration electromigration and electro-osmosis occur in the opposite direction and the electro-osmotic flux has to be larger than that of electromigration (ITP) for the sample to reach the detector. Single-wavelength data for BSA obtained in a 50 μm I.D. capillary with no HPMC and in a 75 μm I.D. capillary with HPMC are depicted in Fig. 5A and B, respectively. In both instances BSA formed a concentration plateau and eluted rapidly as a result of strong electro-osmotic flows.

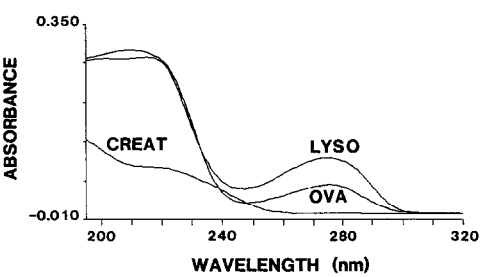

Fig. 3. Time slices of OVA, LYSO and CREAT extracted from the plateau zones of Fig. 2B.

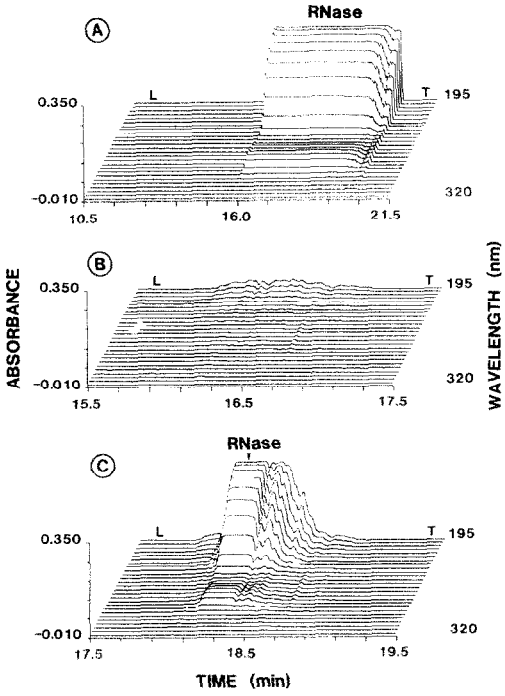

Fig. 4. Cationic isotachopherograms (75 μm I.D. capillary) in the presence of HPMC after (A) the injection of RNase (90 s) (B) a 1% (w/v) solution of pH 3.5–10 ampholine (30 s) and (C) RNase and ampholine (30 s each).

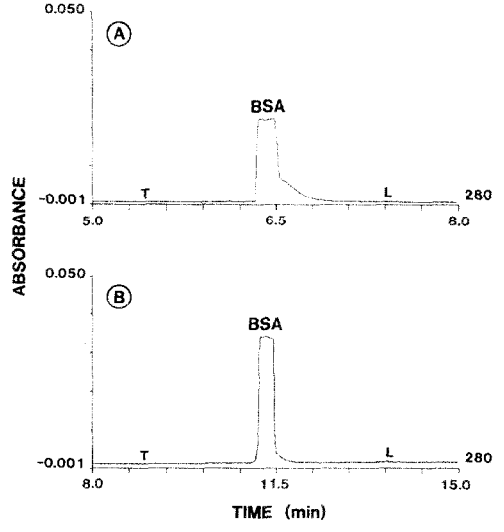

Fig. 5. Anionic ITP of BSA in (A) a 50 μm I.D. capillary without addition of HPMC and (B) in a 75 μm I.D. capillary with HPMC. The initial and final currents were 1 and 4, and 1 and 8 μA, respectively. Only pherograms obtained at 280 nm are displayed.

The difference in magnitude of absorbance is attributed to the difference in I.D. of the capillaries used. Comparison of zone shape reveals that the addition of the polymer does not drastically change the ITP behaviour of BSA, but prevents the formation of the tailing shown in panel A. Note that the rear ITP sample boundary (which passes the detector first) exhibits no tailing. This indicates that the observed dispersion is due to the net zone displacement towards the detector. Clearly, as a result of charge repulsion, anionic protein zones exhibit less interactions with the negatively charged fused-silica surface than the cationic proteins discussed earlier.

CONCLUSIONS

HPMC is believed to form a dynamic coating which drastically reduces the interaction between proteins and the walls as well as the electro-osmotic flow. The polymer is added to the leader in both cationic and anionic analyses so that the coating is renewed for each run. It provides a simple conditioning effect which allows the ITP analysis of many proteins (at the nanogram level) in plain open-tubular fused-silica capillaries of small I.D., *i.e.* in presence of an electro-osmotic flow. Fast-scanning polychrome detection is an interesting approach for the characterization (purity control) of protein zones.

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