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Note

Cationic capillary isotachophoresis of proteins

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Capillary isotachophoresis (ITP)[1,2] is capable of separating and quantitating a wide variety of ionic species, from metals and inorganic anions[3] to synthetic polyelectrolytes[4], peptides and proteins[5]. Proteins normally are separated as anions using high pH electrolytes. Methods for the determination of serum and cerebrospinal fluid proteins[6] and serum lipoproteins[7] have been published. Reports of analyses for proteins and peptides as cations are limited, and include methods for bradykinin and kallidin[8], mouse monoclonal antibody to transferrin[9] and a porcine intestinal peptide with a somatostatin end-group[10].

Our experience with anionic ITP analysis indicates that obtaining an anion with sufficiently low mobility to terminate a protein zone can be difficult. Bocek *et al.*[11] showed that in cationic ITP, H^+ can assume an extremely low effective mobility if a weak acid counter anion is used. Using H^+ as a terminating ion and a weak acid leading buffer, an electrolyte system with a wide mobility range is obtained.

Recently, capillary zone electrophoresis (CZE) in untreated microbore fused-silica tubing showed high-resolution separations of model proteins[12]. Both ITP and CZE offer rapid, instrumental methods of performing electrophoretic separations with on-line detection. One advantage of ITP is highly developed instrumentation which has been commercially available for over a decade. Also, the theory and quantitative aspects of ITP have been extensively studied[13]. Finally, fully automated ITP has been demonstrated[14].

This paper describes the isotachophoretic migration of model proteins in a cationic electrolyte with H^+ termination. Results of quantitative calibrations and separations of mixtures are presented.

EXPERIMENTAL

ITP was performed using a 2127 Tachophor (LKB, Bromma, Sweden) with a 200 × 0.8 mm PTFE capillary. An LKB 2127-140 conductivity/UV detector was used at 280 nm. The leading electrolyte was 10 m*M* potassium acetate adjusted to pH 4.5 with 10% acetic acid, and the terminating electrolyte was 10 m*M* acetic acid. Initial currents were 250 uA for 11 min, at which time the current was reduced to 50 uA for detection. After current reduction, UV and conductivity signals were recorded on a BD-41 strip chart recorder (Kipp and Zonen, Bohemia, NY, U.S.A.) at 3 cm/min; the differential conductivity signal was processed using an ITP data system described previously[15].

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Proteins were obtained from Sigma (St. Louis, MO, U.S.A.) and were diluted to approximately 5 mg/ml in leading electrolyte prior to injection. The 1% ampholyte spacer solution was prepared by diluting 5 μl of pH 3–10 LKB Ampholines® with 200 μl distilled, deionized water. Other chemicals were reagent grade and used without further purification.

RESULTS AND DISCUSSION

Model proteins investigated are listed in Table I, along with molecular weights and pI values. All proteins with pI > 4.5 migrated successfully in the potassium acetate–acetic acid electrolyte system. Pepsin, with a pI of 3.0, failed to migrate as a cation.

Typical conductivity and UV traces for several proteins are shown in Fig. 1 for injections of approximately 10 μg protein. A concentration-regulated sample zone will display a constant-conductance step in the conductivity signal, as can be seen in the pherograms for lysozyme, trypsinogen and β-lactoglobulin A. A zone for Na^+ also is seen in all runs, either as an electrolyte impurity or from the sample. For ovalbumin, the protein mobility is lower than the terminator mobility in their respective zones. Ovalbumin thus migrates as an "enforced" zone[16], and its conductance signal is contained in the overshoot prior to the terminator.

A common measure of effective mobility in ITP systems is the relative step height, or RSH,

$$\mathrm{RSH} = (h_X - h_L)/(h_T - h_L) \quad (1)$$

where h_X, h_L and h_T are the uncalibrated conductivity levels in the sample, leading and terminating zones respectively. RSH values for the ten model proteins tested are

TABLE I

MOLECULAR WEIGHTS, pI VALUES, RELATIVE STEP HEIGHTS AND UV ZONE TRANSMITTANCES OF MODEL PROTEINS

nsm = not sufficiently mobile.

Protein (origin)	*Mol. wt. (ref. 12)*	*pI (ref. 12)*	*RSH*	*%T*
Lysozyme (chicken egg white)	14 000	11	0.52	24
Cytochrome *c* (horse heart)	13 000	9.4	0.53	31
Ribonuclease A (bovine pancreas)	13 500	9.3	0.71	70
Trypsinogen (bovine pancreas)	24 000*	9.3*	0.85	30
Myoglobin (horse heart)	17 500	8.1	0.57	30
Conalbumin (chicken egg white)	77 000	6.6	0.95	31
Carbonic anhydrase (bovine erythrocyte)	29 000*	5.9*	0.85	27
β-Lactoglobulin B (bovine milk)	35 000	5.2	1.03	36
β-Lactoglobulin A (bovine milk)	35 000	5.1	1.05	35
Ovalbumin (chicken egg)	43 500	4.7	1.5	36
Pepsin (hog stomach mucosa)	34 700*	3.0**	nsm	–

* Ref. 19.
** Ref. 20.

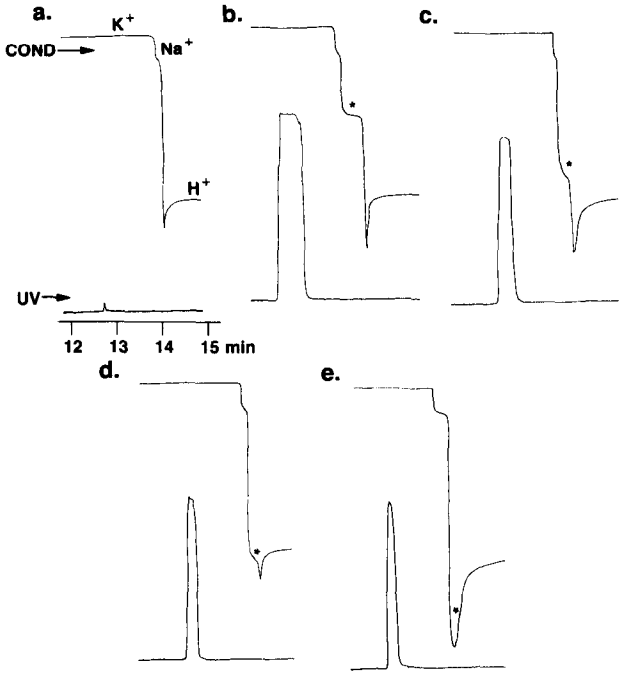

Fig. 1. Conductivity (upper trace) and UV transmittance (lower trace) pherograms for (a) electrolyte blank, (b) 11.4 μg lysozyme, (c) 10.3 μg trypsinogen, (d) 10.2 μg β-lactoglobulin A and (e) 9.6 μg ovalbumin. (*) indicates location of protein zone on conductivity signal. Leading (K^+) and terminating (H^+) zones noted. Time offset in conductivity and UV signals is due to the physical separation of these detectors in the capillary. For conditions, see Experimental section.

given in Table I. A significant correlation between p*I* value and effective mobility in pH 4.5 electrolytes is seen, although the molecular weight obviously plays an important role (*cf.* conalbumin *vs.* carbonic anhydrase). In addition, the particular amino acid composition and conformation of a protein will affect its free solution mobility.

UV signals shown in Fig. 1 display the usual "plateau" shape of concentration-regulated ITP zones which have lengths longer than the detector slit width (*ca.* 0.3 mm). These signals reveal that the proteins are properly stacked between leader and terminator. The Tachophor UV detector output is in transmittance (T), and the transmittance value at the plateau is given in Table I for each protein. All proteins except ribonuclease A display similar %T levels at 280 nm.

As a test of the steady-state ITP migration of these proteins, calibration curves were constructed by injecting varying amounts of high and low mobility proteins, lysozyme and ovalbumin. Table II summarizes the calibration curves obtained by plotting zone length in seconds *vs.* μg injected for both conductivity and UV detection of these proteins. Conductivity zone lengths were determined from the differential signal for lysozyme, or from the overshoot width at RSH = 1.25 for ovalbumin. UV zone lengths were measured manually as plateau widths at half height. Good linearity is seen from 5 to 50 μg injected for both proteins using either detector.

TABLE II

CALIBRATION DATA FOR UV AND CONDUCTIVITY DETECTION OF LYSOZYME AND OVALBUMIN

Lysozyme: range (μg) = 5.7–51.1; n = 7. Ovalbumin: range (μg) = 4.8–43.2; n = 5.

	Lysozyme		*Ovalbumin*	
	UV	*Cond.*	*UV*	*Cond.*
Slope (s/μg)	2.26	2.09	1.23	1.81
Intercept (s)	0.9	0.6	−0.9	−1.1
Corr. coeff.	0.9997	0.9971	0.9998	0.9970

To determine the detection limit for lysozyme in the present system, successively smaller amounts of protein were injected and the resulting UV signals are shown in Fig. 2. Based on 2.5-times the peak height from the electrolyte blank, the detection limit is calculated to be approximately 30 ng. For a 10-μl injection volume, this corresponds to a lower concentration limit of detection of 3 ppm.

ITP is appealing as a quick, quantitative method for separating mixtures of proteins. For small ions with sharp zone boundaries, differences in RSH as small as 0.02 often will yield a complete separation. Separations of a model protein mixture of lysozyme, ribonuclease A, trypsinogen, β-lactoglobulin A and ovalbumin are shown in Fig. 3. Complete separation of this mixture is indicated from the conductivity signal. The UV trace confirms this separation, and proportional increases in zone lengths with amount injected indicate that the ITP steady-state has been reached. However, due to similarities in zone transmittances, discernment of individual protein zones from the UV signal is difficult.

Occasionally, a UV absorbing mixture exhibits plateau separation due to non-UV absorbing impurities migrating at the zone boundaries. The present cationic electrolyte system is very pure (except for Na^+) and lacks sufficient spacer ions for baseline protein separations on the UV signal. Ampholytes commonly are added in anionic protein ITP to obtain spatial separation of UV absorbing zones[17]. Fig. 4 shows the UV and conductance signals for a 0.5-μl injection of a 1% solution of pH

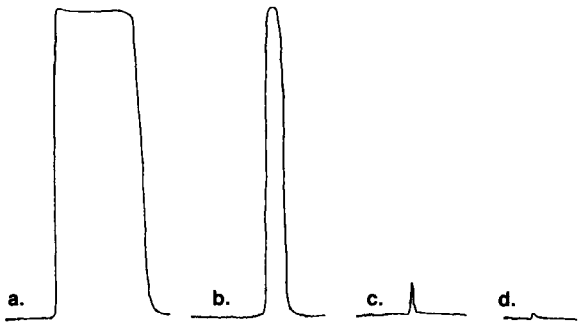

Fig. 2. UV signal for injection of (a) 28.4, (b) 5.7, (c) 0.06 and (d) 0 μg lysozyme.

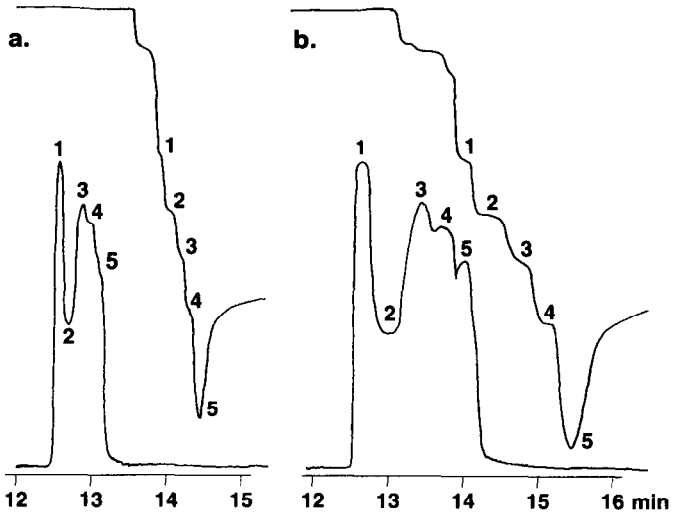

Fig. 3. Conductivity (upper trace) and UV (lower trace) pherograms for (a) 4-μl injection and (b) 10-μl injection of a mixture containing (1) 5.7 mg/ml lysozyme, (2) 5.8 mg/ml ribonuclease A, (3) 5.1 mg/ml trypsinogen, (4) 5.1 mg/ml β-lactoglobulin A and (5) 4.8 mg/ml ovalbumin.

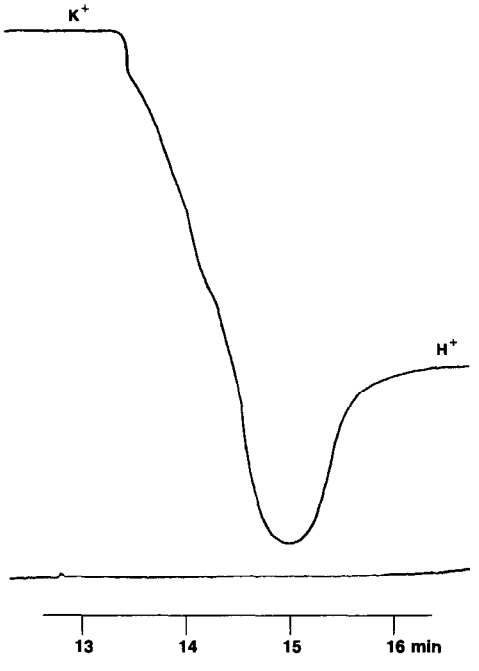

Fig. 4. Conductivity (upper trace) and UV (lower trace) pherograms for a 0.5-μl injection of 1% ampholyte, pH 3–10.

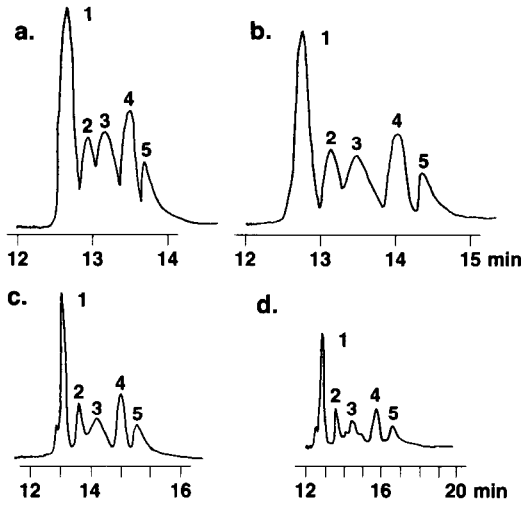

Fig. 5. UV transmittance records of injections of 2 μl of the mixture from Fig. 3, plus (a) 0.5 μl, (b) 1 μl, (c) 2 μl and (d) 4 μl 1% ampholyte spacer. Recorder chart speed is (a,b) 3, (c) 1, and (d) 0.5 cm/min.

3–10 ampholytes. The gradual slope in the conductivity trace indicates a relatively continuous distribution of ampholyte mobilities between leader and terminator. This distribution shows that wide range ampholytes are appropriate for spacing protein mixtures in cationic electrolytes. Separations of the above mixture using varying amounts of ampholytes is shown in Fig. 5. Addition of ampholytes decreases the separation capacity of the system by introducing additional ionic species which must be separated from the sample. Complete separation usually is impossible and sample zones will be mixed and diluted with ampholyte species. Protein zones in Fig. 5a are broader and less absorbing than in runs without spacers. Nevertheless, this mixture of five proteins is resolved in 15 min. Fig. 5b–d show the effect of injecting increasing amounts of ampholytes. With 2 and 4 μl ampholytes added, minor peaks appear before lysozyme and on either side of trypsinogen. The resolution apparently increases, but at a cost in sensitivity and analysis time.

Experiments were performed with mixtures of all ten proteins listed in Table I. At best, seven major peaks could be observed in the pherograms. Using the present electrolyte system, a difference in RSH of approximately 0.1 is necessary to ensure complete resolution. While longer capillaries could yield improved separations, use of discrete, non-UV absorbing spacer cations[18] is more promising. Addition of well-defined mixtures of spacers can reduce sample/spacer zone mixing and result in sharper zones with higher sensitivities, similar to those seen in Fig. 1. Development of such cationic spacers is in progress.

These results indicate that cationic ITP with H^+ termination is well-suited to separating a variety of proteins. Separation of simple mixtures (*e.g.* assaying a protein in a formulation) could be performed rapidly and with little sample preparation. Extension of the method to more complex matrices, such as biological fluids, will require improved spacer mixtures and optimized conditions.

REFERENCES

1 F. M. Everaerts, J. Beckers and Th. P. E. M. Verheggen, *Isotachophoresis — Theory, Instrumentation and Applications* (*J. Chromatogr. Library Ser.*, Vol. 6), Elsevier, Amsterdam, 1976.
2 P. Boček, P. Gebauer, V. Dolník and F. Foret, *J. Chromatogr.*, 334 (1985) 157.
3 P. Boček and F. Foret, *J. Chromatogr.*, 313 (1984) 189.
4 L. R. Whitlock, in J. W. Jorgenson and M. Phillips (Editors), *New Directions in Electrophoresis* (ACS Symp. Ser., No. 335), American Chemical Society, Washington, DC, 1985.
5 S.-G. Hjalmarsson and A. Baldesten, *CRC Crit. Rev. Anal. Chem.*, 11 (1981) 261.
6 P. Delmotte, *Sep. Purif. Methods*, 10 (1981) 21.
7 G. Schmitz, U. Borgmann and G. Assmann, *J. Chromatogr.*, 320 (1985) 253.
8 H. Miyazaki and K. Katoh, *J. Chromatogr.*, 119 (1976) 369.
9 V. Kašička and Z. Prusík, *J. Chromatogr.*, 273 (1983) 117.
10 L. Pradayrol, J. A. Chayvialle, M. Carlquist and V. Mutt, *Biochem. Biophys. Res. Commun.*, 85 (1978) 701.
11 P. Boček, P. Gebauer and M. Deml, *J. Chromatogr.*, 219 (1981) 21.
12 H. H. Lauer and D. McManigill, *Anal. Chem.*, 58 (1986) 166.
13 F. E. P. Mikkers, F. M. Everaerts and J. A. F. Peek, *J. Chromatogr.*, 168 (1979) 293.
14 F. S. Stover, K. L. Deppermann, W. A. Grote and D. V. Vinjamoori, *J. Chromatogr.*, 390 (1987) 61.
15 F. S. Stover, K. L. Deppermann and W. A. Grote, *J. Chromatogr.*, 269 (1983) 198.
16 P. Gebauer and P. Boček, *J. Chromatogr.*, 267 (1983) 49.
17 Reference 1, pp. 322–335.
18 S. Husmann-Holloway and E. Borriss, *Fresenius Z. Anal. Chem.*, 311 (1982) 465.
19 *Biochemicals and Organic Compounds Catalog*, Sigma Chemical Co., St. Louis, MO, 1987.
20 P. G. Righetti, G. Tudor and K. Ek, *J. Chromatogr.*, 220 (1981) 115.