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ION SELECTIVITY IN THE HIGH-PERFORMANCE CATION-EXCHANGE CHROMATOGRAPHY OF PROTEINS

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SUMMARY

The effects of varying the ionic composition of some aqueous mobile phases were observed during the analysis of five proteins by gradient elution on a weak cation-exchange column, SynChropak CM 300. Changes in both selectivity and band broadening occurred when either cations or anions were varied, contrary to similar studies for small molecules. Cations generally follow the same order for retentive properties for proteins as they do for small molecules. However, anions affect the retention of proteins in an inverse order from the way they affect small molecules on anion-exchange.

INTRODUCTION

During the past five years, high-performance ion-exchange chromatography has become increasingly popular for the analysis of proteins[1,2]. Most ion-exchange analyses have been performed with salt gradients of either sodium chloride or sodium acetate with little attention given to solvent composition. Resolution differences for trypsin and chymotrypsin were seen when mobile phase composition was varied on a weak cation-exchanger, giving preliminary evidence that salt composition was an important chromatographic parameter[3]. Recently, Regnier *et al.* examined some effects of anion and cation choice on two proteins analyzed on strong anion- and cation-exchange columns[4]. These reports suggest that mobile phase composition may provide a new dimension to ion-exchange that would allow a multitude of separations on a single column. Such studies have been carried out for small molecules such as nucleosides[5,6] and could be equally beneficial for planning protein analyses.

This study examines the effects of ten salts on elution characteristics such as selectivity and band-broadening for five proteins using a weak cation-exchange support. Both retention plots and chromatograms of protein mixtures will be used to illustrate the importance of ionic composition on selectivity and resolution.

EXPERIMENTAL

Chemicals

Tris (hydroxymethyl)aminomethane (Tris), ammonium chloride, and sodium

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acetate were purchased from Aldrich (Milwaukee, WI, U.S.A.). Sodium chloride and potassium chloride were from American Scientific Products (McGaw Park, IL, U.S.A.). Magnesium chloride, calcium chloride, barium chloride, sodium sulfate, sodium dihydrogen phosphate, 3-(N-Morpholino)propanesulfonic acid sodium salt (MOPS), lysozyme, ribonuclease A, α-chymotrypsin, chymotrypsinogen A, cytochrome *c*, trypsin, and N-benzoyl-DL-arginine *p*-nitroanilide (BAPNA) were all obtained from Sigma (St. Louis, MO, U.S.A.).

Apparatus

The SynChropak CM300 column, 250 × 4.1 mm I.D., was obtained from SynChrom (Linden, IN, U.S.A.). A Varian Model 5000 gradient high-performance liquid chromatograph with a Valco Model CV-6-UHPa-N-60 injection valve (Varian, Walnut Creek, CA, U.S.A.) and an AN-203 UV detector (Anspec, Ann Arbor, MI, U.S.A.) with a Linear Model 1200 recorder (Linear Instruments, Irvine, CA, U.S.A.) were used for the analyses. Because of the consistency needed for the data comparisons, all parts of the apparatus, including columns and tubing, were the same throughout the experiments.

Methods

The A buffer for all experiments was 0.02 *M* Tris in distilled water, pH adjusted to 7 with hydrochloric acid. The buffer for all salts listed in Table I, except for phosphate, was 0.02 *M* Tris and 0.5 *M* salt in distilled water, pH adjusted to 7 with hydrochloric acid. The sodium phosphate buffer was prepared by adding 0.5 *M* sodium dihydrogen phosphate to 0.02 *M* Tris and adjusting the pH to 7 with sodium hydroxide. The gradients for the salts with monovalent ions went from 0 to 100% B in 30 min. The gradients for salts with divalent ions were changed from 0 to 100% B in 60 min to equalize ionic strengths between the two types of salts. Standards were all dissolved in the A buffer.

RESULTS

Nature of the cation

When small molecules are analyzed by cation-exchange chromatography, the retention increases with the use of different cations in the following manner[7]: $Ba^{2+} < Ca^{2+} < Mg^{2+} < K^{+} < NH_4^{+} < Na^{+}$. The displacing powers of a series of cations on the elution of ribonuclease A, cytochrome *c*, α-chymotrypsin, chymotrypsinogen A and lysozyme are seen in Fig. 1. It can be seen that the general trend for displacement of small molecules is followed. Two exceptions are: the order for K^{+} and NH_4^{+} is switched for all proteins except chymotrypsinogen, and lysozyme shows a deviation with Mg^{2+}. This plot also illustrates the separation potential for the series of proteins by each salt. Only $BaCl_2$, $MgCl_2$, and NH_4Cl can separate all of the proteins; in addition, selectivity for different protein pairs varies drastically. A measure of this selectivity can be seen by examining the ratio of retention times for different pairs of proteins. Table I shows such calculations based on lysozyme as a reference. There is more variation for the selectivities of the cations for cytochrome *c* than for chymotrypsinogen. These variances may reflect the similarity of chymotrypsinogen to lysozyme in terms of solvent effects or the dependence of cytochrome *c* elution on the nature of the cation.

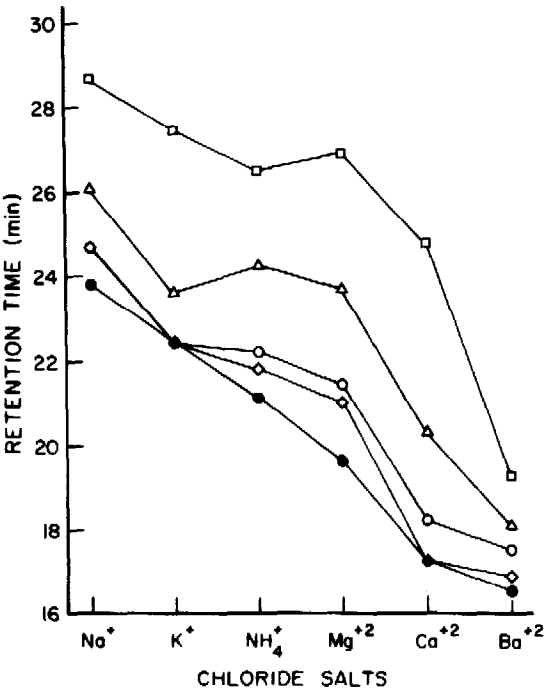

Fig. 1. Effect of cations on elution. Column: SynChropak CM 300, 250 × 4.1 mm I.D. Flow-rates: 1 ml/min. Pressure: 80 atm. Buffer: 0.02 *M* Tris, pH 7, with salt gradient as indicated in Methods. The proteins analyzed were: □, lysozyme; △, chymotrypsinogen A; ○, α-chymotrypsin; ◇, cytochrome *c*; and ●, ribonuclease A.

TABLE I

RELATIVE RETENTION OF PROTEINS ON A WEAK CATION-EXCHANGE COLUMN

Conditions for the analyses were: column, SynChropak CM300, 250 × 4.1 mm I.D.; flow-rate, 1 ml/min; 30- or 60-min gradient, as in text, from 0 to 0.5 *M* salt. Lys = lysine; Ribo = ribonuclease A; Cyt *c* = cytochrome *c*; Chym = α-chymotrypsin; Chymogen = chymotrypsinogen A.

Ion	*Retention ratio*			
	Lys/Ribo	*Lys/Cyt c*	*Lys/Chym*	*Lys/Chymogen*
Anions (sodium salt)				
MOPS	1.23	–	1.11	1.06
Acetate	1.33	1.19	1.19	1.08
Chloride	1.20	1.16	1.16	1.10
Sulfate	1.42	1.13	1.16	1.07
Phosphate	1.33	–	1.17	1.14
Cations (chloride salt)				
Sodium	1.20	1.16	1.16	1.10
Potassium	1.22	1.22	1.22	1.16
Ammonium	1.25	1.21	1.19	1.09
Magnesium	1.37	1.28	1.25	1.13
Calcium	1.43	1.43	1.35	1.21
Barium	1.16	1.13	1.10	1.06

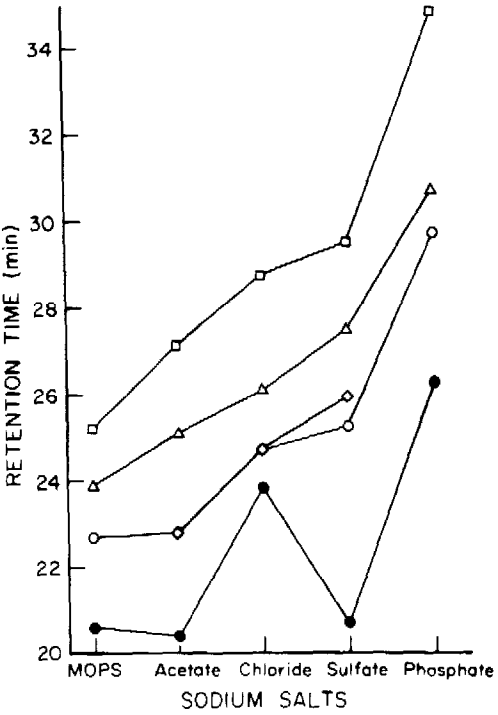

Fig. 2. Effect of anions on elution. Conditions as in Fig. 1. The proteins analyzed were: □, lysozyme △, chymotrypsinogen A; ○, α-chymotrypsin; ◇, cytochrome *c*; and ●, ribonuclease A.

TABLE II

THE EFFECT OF SALT ON BAND BROADENING FOR PROTEINS

Conditions as with Table I.

Ion	*Retention time/peak width*				
	Ribo	*Cyt c*	*Chym*	*Chymogen*	*Lys*
Anions (sodium salts)					
MOPS	44.8	–	25.2	22.3	26.4
Acetate	64.4	42.8	28.1	21.7	34.1
Chloride	51.6	51.9	26.5	24.9	30.9
Sulfate	30.8	31.7	29.9	34.5	48.3
Phosphate	65.8	–	28.3	31.4	27.3
Cations (chloride salts)					
Sodium	51.6	51.9	26.5	24.9	30.9
Potasium	40.2	41.4	22.9	15.4	36.3
Ammonium	45.8	51.4	23.8	25.1	38.5
Magnesium	38.8	30.2	17.6	24.1	40.0
Calcium	30.1	30.1	21.1	15.1	13.4
Barium	41.8	41.6	29.1	28.3	21.8

Nature of the anion

The retention times for small molecules increase in anion-exchange chromatography according to the following order of anions: sulfate < chloride < acetate. Small molecules are not affected by a change of anions in cation-exchange chromatography[8]. The effects of different anions on cation-exchange chromatography of proteins are seen in Fig. 2. Except for ribonuclease A, all of the proteins are eluted in the reverse order to that expected for small molecules with sulfate, chloride, and acetate on anion exchange. The data are more complex than those in Fig. 1 because not all of the salts are totally ionized at pH 7. MOPS is only partially ionized, and phosphate contains two anionic forms. Nonetheless, an idea of the general trends and the ion selectivity can be gained from the plot. Only sodium sulfate totally resolves the five proteins in this study. When the selectivities relative to lysozyme are examined in Table I, much less variance is seen than was observed for the chloride salts. Despite the differences in ionic strengths resulting from the use of salts of weak acids, only small changes in selectivity occur for cytochrome *c*, α-chymotrypsin, and chymotrypsinogen A. Ribonuclease A has more variance, resulting primarily from the radical effects of sodium sulfate on its elution.

Effects of ion composition on band-spreading

Each of the salts in this study had some effect on the peak widths of the proteins, but these effects were not consistent for all proteins. Because proteins must be eluted with a gradient, ordinary plate height measurements cannot be used; therefore, the ratios of retention time/peak width have been calculated for the proteins in the various salt systems and are listed in Table II. This ratio produces larger numbers for narrower peaks. The widths of some proteins, such as chymotrypsin, are barely

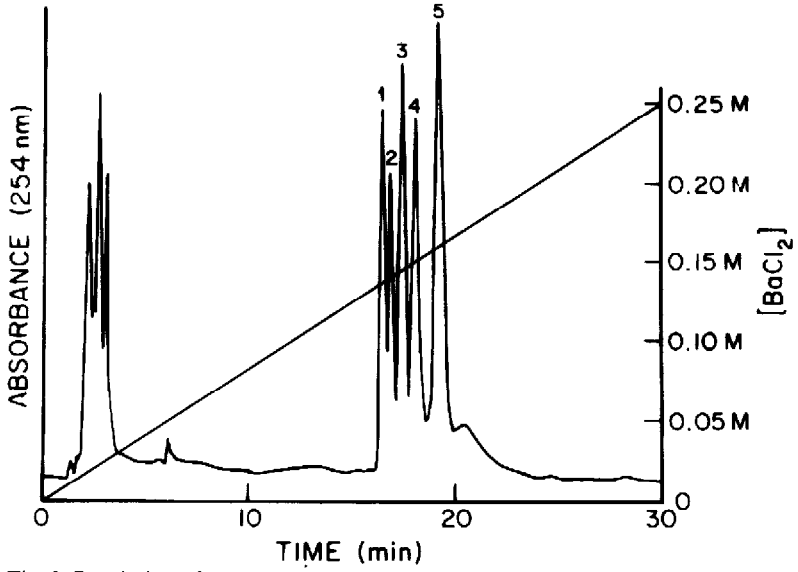

Fig. 3. Resolution of a protein mixture with barium chloride. Column: SynChropak CM 300, 250 × 4.1 mm I.D. Flow-rate: 1 ml/min. Pressure: 80 atm. 60-min gradient from 0 to 0.5 *M* barium chloride in 0.02 *M* Tris, pH 7. Ribo (1), Cyt *c* (2), Chym (3), Chymogen (4), Lys (5).

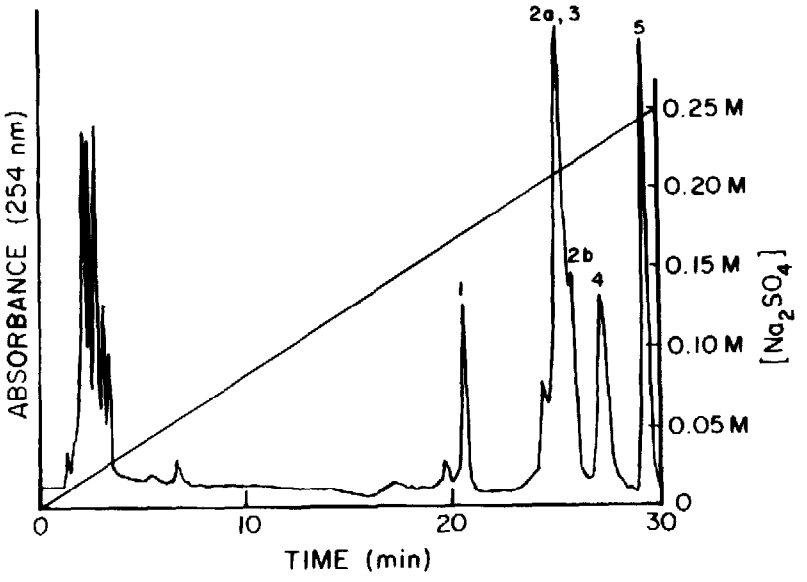

Fig. 4. Resolution of a protein mixture with sodium sulfate. Column: SynChropak CM 300, 250 × 4.1 mm I.D. Flow-rate: 1 ml/min. Pressure: 80 atm. 60-min gradient from 0 to 0.5 *M* sodium sulfate in 0.02 *M* Tris, pH 7. Proteins as in Fig. 3.

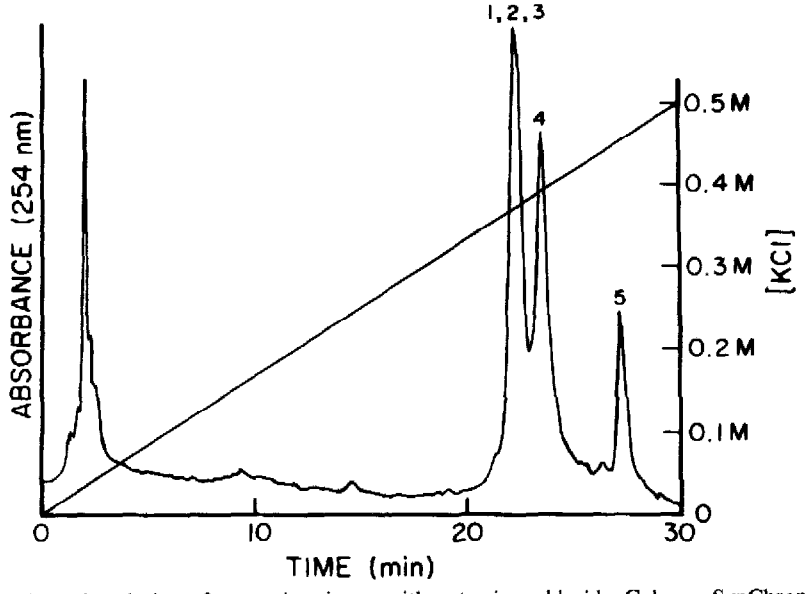

Fig. 5. Resolution of a protein mixture with potassium chloride. Column: SynChropak CM 300, 250 × 4.1 mm I.D. Flow-rate: 1 ml/min. Pressure: 80 atm. 30-min gradient from 0 to 0.5 *M* potassium chloride in 0.02 *M* Tris, pH 7. Proteins as in Fig. 3.

affected by salt composition, whereas ribonuclease A yields a narrow peak in phosphate, but a broad one in sulfate.

DISCUSSION

Several conclusions can be drawn from the results presented here. In general, cations displace proteins from a cation-exchange support in the same order of effectiveness that they displace small molecules. Anions, however, also affect the retention of proteins on cation-exchange columns even though they have no effect on small molecules. The retention characteristics of proteins produced by anions are opposite to those of small molecules on anion-exchange supports. These results are complex, because a protein is composed of amino acids with both anionic and cationic functionalities. Mobile phase salts titrate both the anions on the cation-exchange support and the ions on the protein molecules. Therefore, anions and cations are both important. The effects of salts on the band widths of some proteins are again related to protein structure and uniqueness. This study showed calcium chloride to cause band-spreading for all five proteins, but otherwise there was no general effect on peak width from the use of a particular salt. These unique qualities of individual proteins can be used to enhance a separation on a given support. Figs. 3–7 illustrate the analyses of the five test proteins with five different salts. Although barium chloride (Fig. 3) gives the best overall resolution of the five proteins, sodium sulfate (Fig. 4) best separates ribonuclease A and cytochrome *c*, and potassium chloride (Fig. 5) best

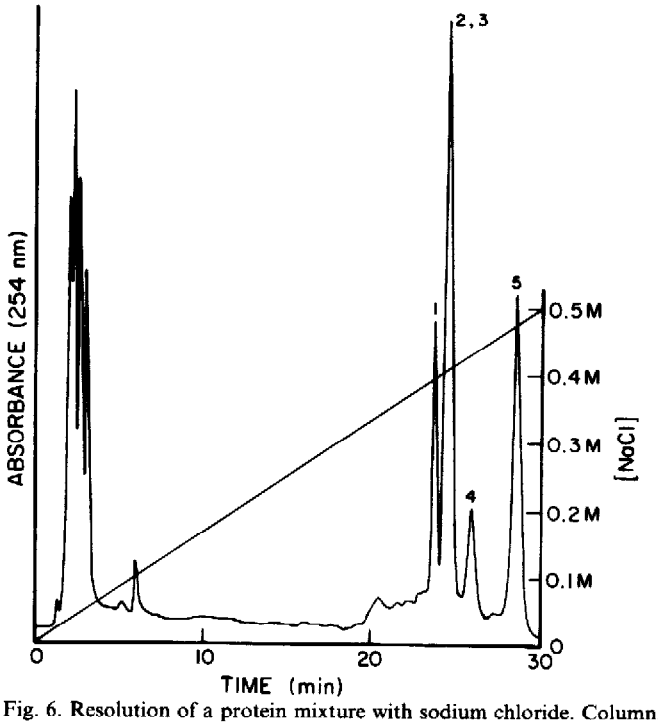

Fig. 6. Resolution of a protein mixture with sodium chloride. Column: SynChropak CM 300, 250 × 4.1 mm I.D. Flow-rate: 1 ml/min. Pressure: 80 atm. 30-min gradient from 0 to 0.5 *M* sodium chloride in 0.02 *M* Tris, pH 7. Proteins as in Fig. 3.

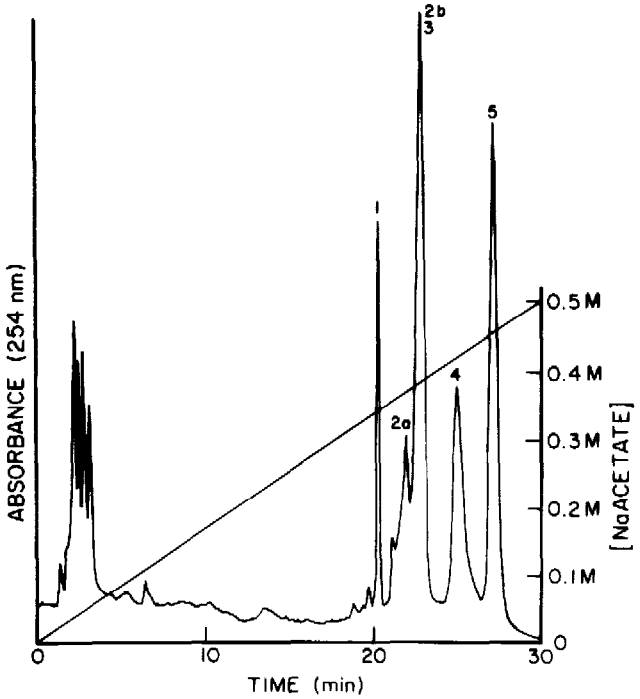

Fig. 7. Resolution of a protein mixture with sodium acetate. Column: SynChropak CM 300, 250 × 4.1 mm I.D. Flow-rate: 1 ml/min. Pressure: 80 atm. 30-min gradient from 0 to 0.5 *M* sodium acetate in 0.02 *M* Tris, pH 7. Proteins as in Fig. 3.

resolves chymotrypsinogen A and lysozyme. As a comparison, analyses with sodium chloride and sodium acetate are shown in Figs. 6 and 7, respectively, since the majority of HPLC analyses by ion-exchange have used these salts. These data suggest that an inadequate separation of proteins on a given ion-exchange column might be made satisfactory by simply changing the salt composition used for gradient elution.

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