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PURIFICATION OF TRYPSIN AND OTHER BASIC PROTEINS BY HIGH-PERFORMANCE CATION-EXCHANGE CHROMATOGRAPHY

KAREN M. GOODING* and MARY NELL SCHMUCK
SynChrom, Inc., P.O. Box 110, Linden, IN 47955 (U.S.A.)

SUMMARY

Conditions for the purification of basic proteins by high-performance cation-exchange chromatography were examined on SynChropak CM 300, a carboxymethyl column packing material. The pH and salt composition of the mobile phase were varied to determine the effect on the retention of trypsin, chymotrypsin, lysozyme and cytochrome *c*. In the course of these studies, the resolution of trypsin and chymotrypsin was optimized so that purification of either enzyme could be effected. Enzyme activity was monitored during the analyses. The capacities of an analytical and a semi-preparative column were determined for chymotrypsin.

INTRODUCTION

One of the purification problems which has continually plagued biochemists is the isolation of trypsin from chymotrypsin. Trypsin must be free of contamination from other proteases to be useful in sequencing studies, but commercial preparations are rarely totally pure. Besides containing other proteins, trypsin standards often contain multiple forms of the enzyme and autolysis products. Various chromatographic methods which have been developed for the purification of trypsin include cation exchange on a sulfoethyl gel column[1], hydrophobic interaction on a methacrylate gel column[2] and reversed-phase chromatography on a cyano column[3]. One purpose of this investigation was to develop a method for the purification of trypsin and chymotrypsin using high-performance cation-exchange chromatography.

Methods of protein analysis using high-performance liquid chromatography (HPLC) in the anion-exchange, steric exclusion and reversed-phase modes have been rapidly growing in number over the past several years[4,5]. Unfortunately, there have been only a limited number of published reports which describe high-performance cation exchange of proteins. This has been due primarily to the previous inavailability of suitable commercial support materials. The investigations which have been published made use of weak or strong cation exchangers on rigid gels[6–8], controlled-porosity glass[9] or silica[10,11]. Our method is based on a weak cation exchanger that is polymerized onto microparticulate porous silica. Effects of mobile phase composition on the retention characteristics of several cationic proteins have been studied to determine whether guidelines for solvent selection can be established.

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EXPERIMENTAL

Chemicals

Tris(hydroxymethyl)aminomethane (Tris), ammonium chloride and ammonium acetate were purchased from Aldrich (Milwaukee, WI, U.S.A.). N-2-Hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) was from Research Organics (Cleveland, OH, U.S.A.). Potassium chloride was from Mallinckrodt (St. Louis, MO, U.S.A.). Trypsin, α-chymotrypsin, lysozyme, cytochrome *c*, sodium acetate, benzoyl-DL-arginine *p*-nitroanilide (BAPNA) and glutaryl-1-phenylalanine *p*-nitroanilide (GPN) were all purchased from Sigma (St. Louis, MO, U.S.A.). The sodium chloride and monobasic potassium phosphate were from Mallinckrodt (Paris, KY, U.S.A.).

Apparatus

SynChropak CM 300 columns, 250 × 4.1 mm I.D. or 250 × 10 mm I.D. (particle size 6.5 μm), and a SynChropak PCR column, 400 × 5.0 mm I.D., were 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 a Chem Research Model 2020 multiple-wavelength detector (Instrumentation Specialties Company, Lincoln, NE, U.S.A.) or an Anspec AN-203 UV detector (Anspec Co., Ann Arbor, MI, U.S.A.) were used for the analyses. A Linear Model 1200 recorder (Linear, Irvine, CA, U.S.A.) was used.

Methods

The mobile phases used are listed in Table I. The buffers were prepared in deionized water using a sodium hydroxide solution or hydrochloric acid to adjust the pH. Standards were dissolved in the initial buffer with the exception of trypsin, which required adjustment to pH 3 to reduce autolysis.

Post-column reaction

SynChropak PCR is a macroparticulate non-porous column material with a hydrophilic layer which is essentially non-reactive to enzymes and substrates[12]. A column packed with SynChropak PCR was connected to the exit of the analytical column with a T-tube, into which a solution of the substrate was pumped at 1 ml/min. The effluents from the reactor were monitored at 410 nm. All reactions were performed at room temperature. The reactor volume was 3.7 ml.

The substrate for trypsin was benzoyl-DL-arginine *p*-nitroanilide (BAPNA), which is hydrolyzed by trypsin to produce *p*-nitroaniline[13]. By heating and stirring, 0.79 mmoles of BAPNA were dissolved in 0.1 *M* potassium phosphate, pH 7. BAPNA remained in solution after cooling to room temperature. It remained colorless throughout the heating and chromatography until hydrolysis by trypsin.

The substrate for chymotrypsin was glutaryl-1-phenylalanine *p*-nitroanilide (GPN) which is hydrolyzed by chymotrypsin to produce *p*-nitroaniline[14]. 0.8 mmoles of this substrate were dissolved in 0.1 *M* potassium phosphate, pH 7. Heating and stirring were necessary to effect solution but they did not cause hydrolysis of the substrate.

RESULTS AND DISCUSSION

Mobile phase selection

One of the most critical parameters in HPLC is mobile phase composition. In some chromatographic modes such as adsorption or reversed-phase partition, general guidelines have been established as a result of numerous investigations[15]. Because high-performance ion-exchange chromatography of proteins is still in its infancy, there has been very little research in solvent selection. Fig. 1 illustrates the effect of changing an anion from chloride to acetate in the resolution of the two common forms (reduced and oxidized) of cytochrome *c* by cation-exchange chromatography. Although the acetate ion results in longer retention times and greater selectivity, the chloride ion produces much narrower bands. Table I is a compilation of retention data for four proteins using a variety of salts and pH values. All of these systems implemented a 30-min gradient from 0 to 0.5 *M* salt, a flow-rate of 1 ml/min and a SynChropak CM 300 column, 250 × 4.1 mm I.D. This is a weak cation exchanger having carboxymethyl groups incorporated into a polymeric matrix, which is bonded onto 300-Å silica.

Although the nature of the cation is one of the most important elution parameters in cation-exchange chromatography of small molecules[16], Na^+, NH_4^+ and K^+ had about the same effect on the retention times of these four proteins. A decrease

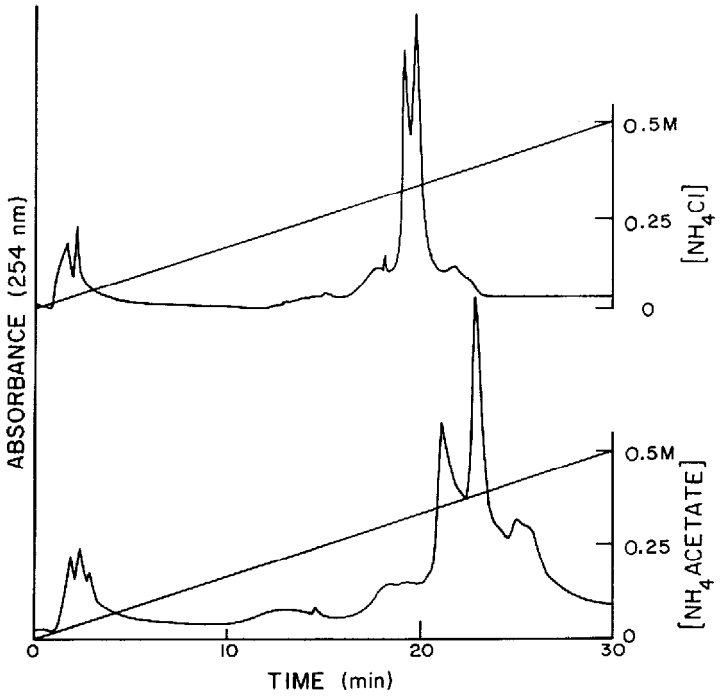

Fig. 1. Effect of the nature of the anion on the resolution of reduced and oxidized cytochrome *c*. Column: SynChropak CM 300, 250 × 4.1 mm I.D. Flow-rate: 1 ml/min. Pressure: 78 atm. 30-min linear gradient from 0 to 0.5 *M* salt. Buffer: 0.02 *M* Tris, pH 7. Salts were either NH_4Cl (top) or $CH_3CO_2NH_4$ (bottom). First peak, reduced form; second peak, oxidized form of cytochrome *c*.

TABLE I

EFFECT OF SALTS AND pH ON RETENTION

Conditions for the analyses were as follows: column, SynChropak CM 300, 250 × 4.1 mm I.D.; 30 min gradient from 0 to 0.5 *M* salt; flow-rate 1 ml/min; pressure 78 atm.

Buffer	*Salt*	*pH*	*Cytochrome c*		*Lysozyme*		*Trypsin*		*Chymotrypsin*		Rs_{C+T}
			t_1	t_2	t	Δt	t	Δt	t	Δt	
0.01 *M* KH_2PO_4	NaCl	6.0	19.1	21.0	25.2	1.5	16.0	2.1	17.2	2.1	0.6
0.01 *M* KH_2PO_4	NaCl	5.0					21.4	4.9	23.7	1.8	0.5
0.02 *M* HEPES	NaCl	7.6	20.8	23.3	24.9	1.6	15.6	0.9	16.8	1.0	1.2
0.02 *M* HEPES	NaCl	5.5		23.8			18.0	1.4	20.7	3.3	1.4
0.02 *M* KH_2PO_4	NaCl	7.3	20.8	22.6	25.6	2.0	14.1	0.9	15.4	1.2	1.1
0.02 *M* Tris	CH_3CO_2Na	7.0	17.8	19.6	31.0	2.4	16.9	0.7	23.1	1.6	5.5
0.02 *M* Tris	KCl	7.0	19.2	19.8	27.4	1.8	19.3	1.3	19.8	1.0	0.4
0.02 *M* Tris	NaCl	7.0	19.1	19.8	26.4	1.4	18.7	2.2	19.6	0.8	0.6
0.02 *M* Tris	NH_4Cl	7.0	19.3	19.8	27.1	1.2	19.8	1.6	19.5	1.2	0.2
0.02 *M* Tris	$CH_3CO_2NH_4$	7.0	21.6	23.0	32.8	3.3	21.6	3.1	22.7	1.6	0.4
0.02 *M* Tris	CH_3CO_2Na*	5.9				1.0	20.8	1.0	23.2	2.0	1.6
0.02 *M* Tris	CH_3CO_2Na*	5.5	22.7	23.2	36.4	1.4	21.4	1.4	24.2	2.6	1.4

* CH_3CO_2Na is not totally ionized at this pH.

in pH from 7.3 to 5 had the expected effect of increasing retention for trypsin and chymotrypsin, but little effect on the elution of cytochrome *c* or lysozyme. The only parameter which had the same effect on all the proteins in this study was the nature of the anion. Acetate resulted in longer retention times and greater selectivity than chloride. This finding substantiates the recommendation of one gel manufacturer to use anionic buffers with cation exchangers[17].

Purification of trypsin and chymotrypsin

The initial goal in the purification of trypsin and chymotrypsin was to optimize the separation of the two enzymes from each other and from any degradation products which may have been present. Table I shows that the resolution obtained with sodium acetate at pH 7 was better than that obtained with any other mobile phase. The separation of trypsin and chymotrypsin with the pH 7 Tris buffer and an acetate gradient is shown in Fig. 2. Because the resolution was so good, this solvent system was chosen for the purification studies. Since trypsin is prone to autolysis at pH 7, a lower pH should be chosen if quantitative preparation is desired. Chymotrypsin was stable at pH 5–7. There was approximately a 15% decrease in peak width when a 10 mm I.D. column and a lower linear velocity were used, but this was not enough increase in resolution to warrant using the larger column.

The procedure chosen for testing the efficacy of this method for enzyme purification consisted of the collection and rechromatography of fractions of the enzyme peaks. To check for contamination, the fractions were assayed for both tryptic and chymotryptic activity by post-column enzyme detection. The reaction in which BAP-NA is the substrate at room temperature, as described in the Experimental, gave ten times greater sensitivity than detection at 254 nm. There was no cross reactivity with the main chymotrypsin peak within the detection limits. The reaction using GPN

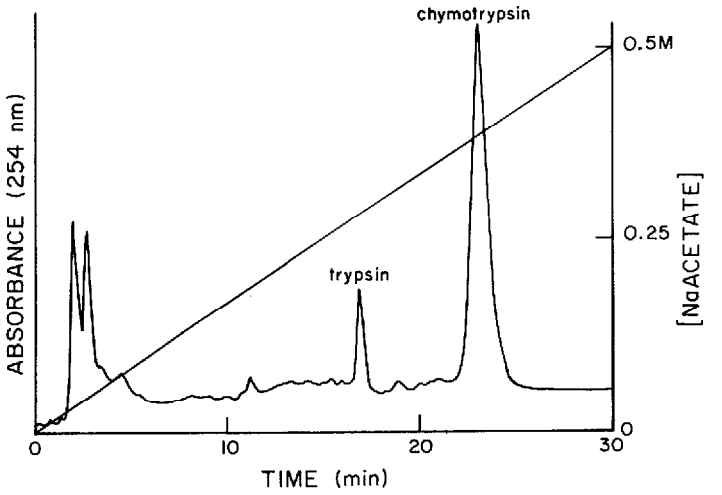

Fig. 2. Resolution of trypsin and chymotrypsin. Column: SynChropak CM 300, 250 × 4.1 mm I.D. Flow-rate: 1 ml/min. Pressure: 78 atm. Buffer: 0.02 *M* Tris, pH 7. 30-min linear gradient from 0 to 0.5 *M* CH_3CO_2Na.

produced no increase in sensitivity over detection at 254 nm under the conditions used; however, the selectivity was increased since there was no cross reactivity with the active trypsin peaks. This selectivity is especially important for identification of the actual enzyme peaks in the case of proteolytic enzymes which undergo autohydrolysis.

In the course of several months, the trypsin sample used in our studies changed, giving rise to many new peaks. Fig. 3a shows the elution profile of this trypsin at 254 nm. Fig. 3b shows the tryptic activity of this trypsin sample with BAPNA as substrate and Fig. 3c illustrates its chymotryptic activity using GPN as substrate. The enzymatic activity profile of trypsin seen in Fib. 3b closely resembles that on SE-Sephadex[1]. Fractions of trypsin were collected in vials containing hydrochloric acid to keep the pH below 3 in order to reduce autolysis. Sample size was 3.3 mg to insure that fractions would be concentrated enough to show contaminants. Detection by the post-column reactor lagged *ca.* 2 min behind UV detection, but the peaks in Figs. 3 and 5 have been aligned for clarity. Fractions A and B each exhibited tryptic activity as seen in Fig. 4. They appear to correspond to the major peak and shoulder of enzymatic activity seen in Fig. 3b. No trace of the earlier trypsin peak was seen in these fractions. There was also no chymotryptic activity in either fraction A or B.

Fig. 5a shows the elution profile of a 2.6-mg sample of chymotrypsin at 254 nm. The enzymatic activity of this chymotrypsin with GPN and BAPNA as substrate for chymotrypsin and trypsin, respectively, are shown in Fig. 5b and 5c. When the fraction denoted as C was rechromatographed it showed a single peak of chymotryptic activity as seen in Fig. 6.

Capacity

Once high-performance cation-exchange chromatography was shown to be suitable for the purification of chymotrypsin and trypsin, it was necessary to determine the load capacity of the column for these proteins. Chymotrypsin was chosen

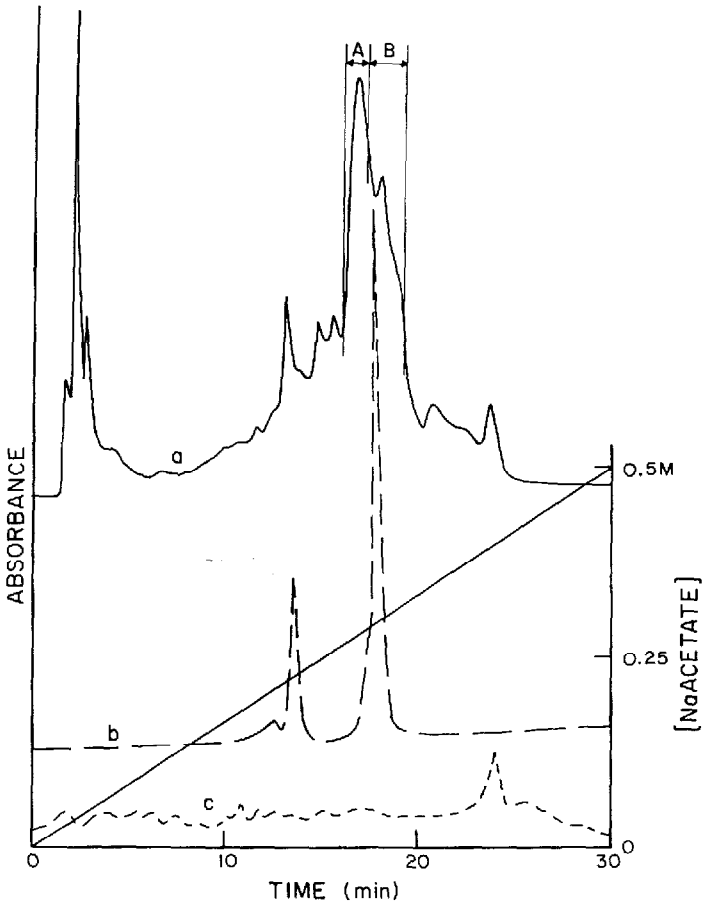

Fig. 3. Analysis of impure trypsin. Conditions as in Fig. 2. In (a), detection at 254 nm. In (b) and (c), detection of enzymatic activity at 410 nm by post-column reaction after chromatography on a Syn-Chropak PCR column, 400 × 5 mm I.D. Substrates were BAPNA in (b) and GPN in (c); both, in 0.1 *M* KH_2PO_4 at pH 7, were pumped at 1 ml/min. Fractions were collected at A and B.

for these studies because its rate of autohydrolysis was much lower than that of trypsin. Fig. 7 illustrates the effect of overloading on the chromatographic profile of chymotrypsin. With 10 mg, the retention time decreased and the peak lost its Gaussian shape, as is typical of overloading. With 20 mg, the peak had become totally flat. Similar loading experiments were conducted on a semi-preparative column that was 250 × 10 mm I.D. A comparison of these results, as seen in Fig. 8, with those on the 250 × 4.1 mm I.D. column seen in Fig. 7 show that the larger column has approximately five times the capacity. This roughly corresponds to the six-fold increase in the amount of packing material in the larger column. Loading experiments with cytochrome *c* on the analytical column gave results nearly identical to those for chymotrypsin.

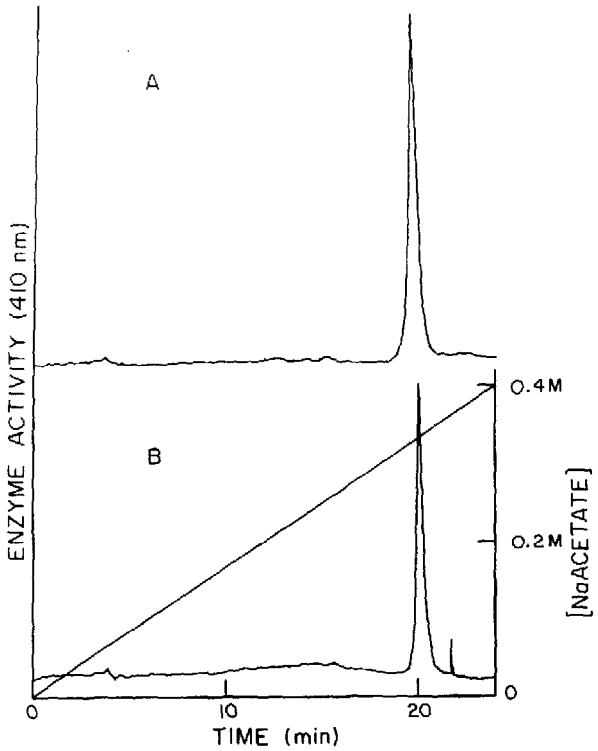

Fig. 4. Rechromatography of fractions A and B from Fig. 3a. Conditions as in Fig. 2. Detection of tryptic activity by post-column reaction with BAPNA as substrate.

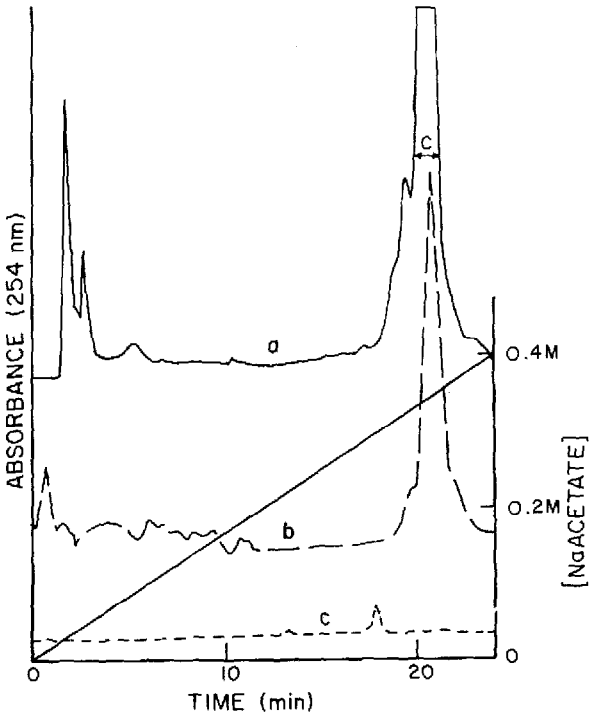

Fig. 5. Analysis of chymotrypsin. Conditions as in Fig. 2. Post-column reaction with GPN as substrate in (b) and with BAPNA in (c). A fraction was collected at C.

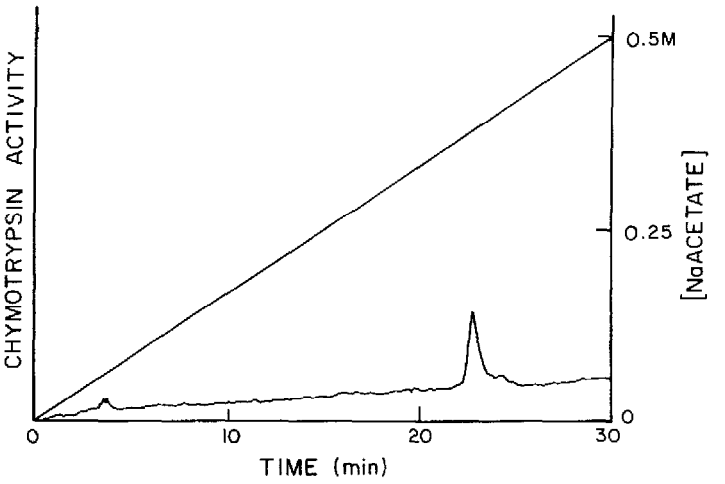

Fig. 6. Rechromatography of fraction C from Fig. 5a. Conditions as in Fig. 2. Conditions for post-column detection as in Fig. 3c, using GPN as substrate for chymotrypsin.

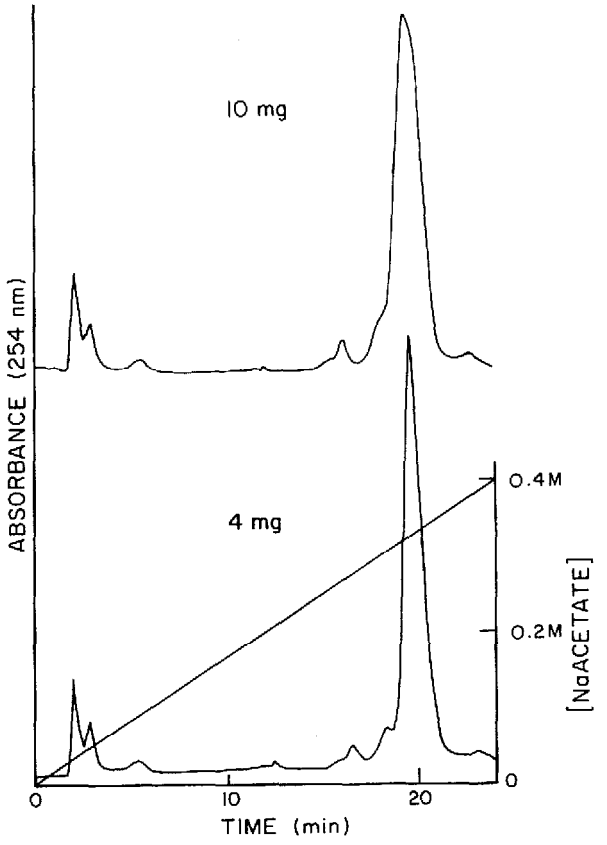

Fig. 7. Effect of sample loading on the resolution of components of a chymotrypsin sample using an analytical column of SynChropak CM 300, 250 × 4.1 mm I.D. Conditions as in Fig. 2.

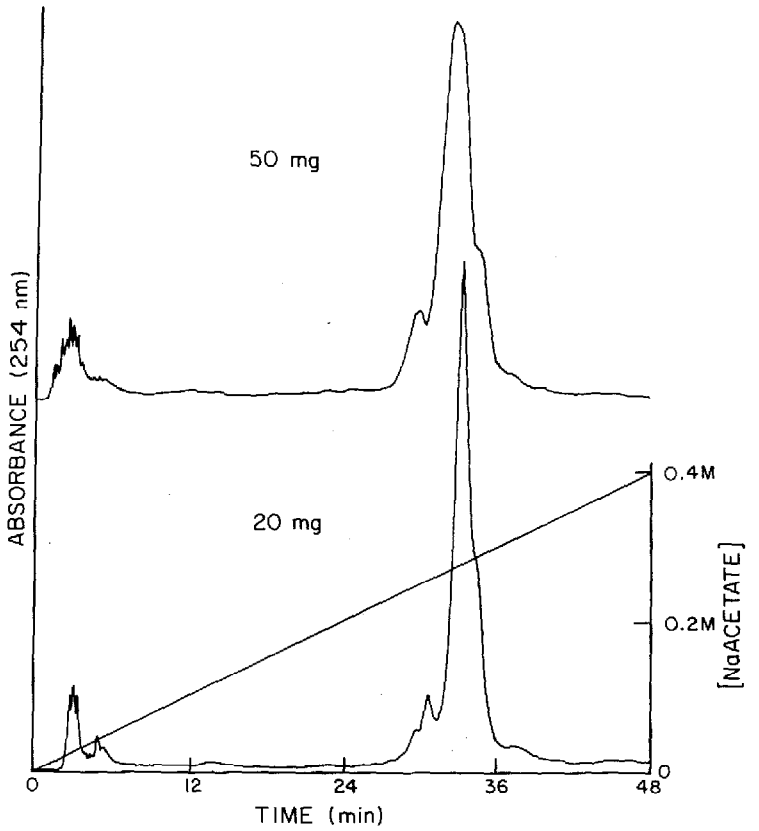

Fig. 8. Effect of sample loading on the resolution of components of a chymotrypsin sample using a semi-preparative column. Column: SynChropak CM 300, 250 × 10 mm I.D. Flow-rate: 3 ml/min. Pressure: 48 atm. Buffer 0.02 *M* Tris, pH 7. 60-min linear gradient from 0 to 0.5 *M* CH_3CO_2Na.

CONCLUSIONS

The anomalous results presented here regarding the mobile-phase effects on cation exchange are characteristic of protein chromatography. Unlike small molecules, which can be easily characterized as to charge or hydrophobicity, proteins are composed of numerous ionic and hydrophobic sites and regions. These moieties can interact with an ion-exchange matrix, buffer, salts or neighboring groups. This complexity makes it difficult to establish general guidelines for any chromatographic mode, but especially for ion exchange. When formulating a new analysis for a protein, it is still best to try several buffer systems to find the one most suited to the purpose of the analysis.

High-performance cation-exchange chromatography is shown to be suitable for the analysis of trypsin or chymotrypsin samples. Our analytical column had a capacity for chymotrypsin of 10 mg and that of our semi-preparative column was 50 mg. The fact that the loading study with cytochrome *c* gave nearly identical results suggests that the capacity may be similar for most cationic proteins with a molecular weight below 30,000.

REFERENCES

1 D. D. Schroeder and E. Shaw, *J. Biol. Chem.*, 243 (1968) 2943.
2 P. Štrop and D. Čechová, *J. Chromatogr.*, 207 (1981) 55.
3 K. Titani, T. Sasagawa, K. Resing and K. A. Welsh, *Anal. Biochem.*, 123 (1982) 408.
4 F. E. Regnier and K. M. Gooding, *Anal. Biochem.*, 103 (1980) 1.
5 M. T. W. Hearn, F. E. Regnier and C. T. Wehr, *Amer. Lab.*, 14 (1982) 18.
6 O. Mikeš, P. Štrop, J. Zbrožek and J. Čoupek, *J. Chromatogr.*, 119 (1976) 339.
7 O. Mikěs, P. Štrop and J. Sedláčková, *J. Chromatogr.*, 148 (1978) 237.
8 Sj. van der Wal and J. F. K. Huber, *Anal. Biochem.*, 105 (1980) 219.
9 S.-H. Chang, R. Noel and F. E. Regnier, *Anal. Chem.*, 48 (1976) 1839.
10 C. A. Frolik, L. L. Dart and M. B. Sporn, *Anal. Biochem.*, 125 (1982) 203.
11 S. Gupta, E. Pfannkoch and F. E. Regnier, *Anal. Biochem.*, 128 (1983) 196.
12 T. D. Schlabach, S. H. Chang, K. M. Gooding and F. E. Regnier, *J. Chromatogr.*, 134 (1977) 91.
13 B. J. Haverback, B. Dyce, H. Bundy and H. A. Edmonson, *Amer. J. Med.*, 29 (1960) 424.
14 M. H. Remy, D. Guillochon and D. Thomas, *J. Chromatogr.*, 215 (1981) 87.
15 L. R. Snyder and J. J. Kirkland, *Introduction to Modern Liquid Chromatography*, Wiley, New York, 1979, Chs. 7 and 9.
16 L. R. Snyder and J. J. Kirkland, *Introduction to Modern Liquid Chromatography*, Wiley, New York, 1979, p. 422.
17 *Sephadex® Ion-Exchangers*, Pharmacia Fine Chemicals, Uppsala, 1977, p. 20.