CHROMSYMP. 1834

Optimization of high-performance liquid chromatographic peptide separations with alternative mobile and stationary phases

PATRICIA M. YOUNG* and THOMAS E. WHEAT

Waters Division of Millipore Corp., 34 Maple Street, Milford, MA 01757 (U.S.A.)

ABSTRACT

Peptides are routinely separated with reversed-phase high-performance liquid chromatography using increasing concentrations of acetonitrile in the presence of trifluoroacetic acid. While these separations may be improved by adjustments of gradient slope or substitutions of different solid-phase chemistries, many mixtures would benefit from systematic optimization of mobile phase components. Tryptic digests of cytochrome *c* from various species were separated on Waters Delta-Pak™ C_{18}. The effects of varying pH as well as the concentration and type of ion-pair reagent were examined. In addition, low pH, ion-suppression/ion-pairing chromatography was inverted using a polymeric reversed-phase column at high pH with alkyl amine ion pairing. Finally, a tryptic digest of cytochrome *c* was resolved by ion-exchange chromatography with a strong cation-exchange high-performance liquid chromatography column. These data suggest a framework for dramatically changing the selectivity of peptide separations, leading to more satisfactory peptide mapping.

INTRODUCTION

Peptide mapping is most commonly performed on silica-based reversed-phase columns using aqueous trifluoroacetic acid (TFA) as a starting eluent. Peptides are retained on the column and eluted as a function of increasing organic solvent, usually acetonitrile containing the same concentration of TFA[1–4]. While this mode of chromatography has proven useful, complex mixtures of peptides commonly obtained in enzymatic digests of high-molecular-weight proteins may include too many different species to provide complete resolution of each peptide. Mixtures of peptides within a single peak are unsuitable for amino acid sequencing or compositional analysis[5]. Alternative reversed-phase packings can provide different selctivities[6], but it may be more practical to optimize the separation by changing the mobile phase operating conditions. Conventional peptide mapping with TFA is performed at approximately pH 2, where the carboxyl functions are largely protonated. In addition, TFA serves as an ion pair to the positively charged amino functions. Alterations in mobile phase components and gradient slope can alter peptide retention and selectivity[7–12];

however, alternative separation mechanisms may lead to greater resolution of complex peptide mixtures[12].

The ion pairing and ion suppression can be inverted at basic pH using high-resolution, polymeric reversed phase packings. At pH 11, amine groups will have suppressed ionization, while carboxyl groups will be negatively charged. An ion pair with the carboxyl group can be formed using alkyl amines, resulting in dramatically different reversed-phase separations.

Alternatively, it is possible to exploit the charge of peptides in an ion exchange separation[13]. Each peptide has an amino and carboxyl terminus, but more importantly, some of the amino acid residues have charged groups that contribute to the net charge of the peptide. Ion exchange offers an entirely different selctivity than reversed phase and can be used to obtain additional information about a peptide mixture.

MATERIALS AND METHODS

Tryptic digestion

Cytochrome c (Sigma, St. Louis, MO, U.S.A.) from bovine, rabbit, chicken and horse heart mitochondria (1 mg/500 μl) were suspended in 0.1 M ammonium bicarbonate (Sigma) buffer, pH 8.0. N-Tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin (Worthington Biochemical, Freehold, NJ, U.S.A.) was dissolved at a concentration of 0.2 mg/1 ml buffer. Trypsin solution (500 μl) was added to the cytochrome c suspensions and incubated for 24 h at 37°C.

Following incubation, trypsin was deactivated by heating at 100°C for 5 min. The digests were separated into aliquots of 100 μl and frozen (-20°C) until chromatographed. Prior to high-performance liquid chromatography (HPLC), digests were diluted 1:10 with aqueous TFA.

HPLC system

Samples were chromatographed on a 625 LC System (Waters Division of Millipore, Milford, MA, U.S.A.) equipped with a column heater and autosampler (Waters Model 712). Samples were analyzed by photodiode array detection (Waters Model 990+) in a wavelength range of 190–425 nm with 1.4 nm resolution. Peptide mixtures were separated on both stainless-steel (150 × 3.9 mm and 150 × 2 mm) and non-metallic (3.9 × 150 mm) columns packed with Delta-Pak™ C_{18}, 300 Å, 5 μm as well as RS-Pak™ DS-613 (150 × 6 mm) and Protein-Pak™ SP-5PW (75 mm × 7.5 mm), all from Waters.

Reagents

Chromatographic eluents were Milli-Q™ water (Millipore, Bedford, MA, U.S.A.) and HPLC-grade acetonitrile (Baker, Phillipsburg, NJ, U.S.A.). Heptafluorobutyric acid and sequanal grades of trifluoroacetic acid and hydrochloric acid were obtained from Pierce (Rockford, IL, U.S.A.). Tetrabutylammonium hydrogen sulfate and sodium chloride were obtained from Sigma.

RESULTS AND DISCUSSION

Gradient slope

Peptide retention times are sensitive to the concentration of organic solvent in the mobile phase, and subtle variations of gradient slope are commonly used to optimize a separation. Reductions in gradient slope or more shallow gradient formation result in greater resolution; however, the peptides elute in a larger volume. A 16 column volume gradient to 60% acetonitrile (1%/min) was compared to a 32 column volume gradient (data not shown). Peak heights were 30–50% greater with the steeper (16 column volume) gradient. The total peak volume was on the order of twice as large with the more shallow gradient (32 column volumes). An optimized chromatogram may be a compromise between resolution and sensitivity. More dilute materials may be difficult to use in subsequent analytical steps because fractions may necessitate concentration. This may result in sample loss with concomitant increase in trace contaminants that may be present in the mobile phase. It is important to consider other options that alter the selctivity of the separation so that resolution can be improved without increasing peak volume.

TFA concentration

TFA is used as a mobile phase modifier in peptide mapping to protonate or ion suppress peptide carboxyl groups at pH 2 while ion pairing to basic functionalities. Changes in TFA concentration primarily affect ion suppression and should alter the selectivity of the separation. The effect of varying TFA concentration can be conveniently tested using the Auto-Blend™ method[14] where one of the four inlet solvent lines contains 1% TFA solution (data not shown). The fraction of the flow taken from the stock TFA solution determined the final concentration blended in the mobile phase without mixing additional starting eluents. Separation of peptides at 0.1% and 0.05% TFA resulted in similar maps with subtle alterations in resolution. Some closely spaced peaks were resolved better with lower concentrations of TFA while other difficult pairs were separated better at higher concentrations of TFA. In addition, the baseline rise was approximately half as great with 0.05% TFA.

Alternative ion-pair reagents

The selective effects of ion-pair reagents were tested by separating a tryptic digest with a linear gradient in the presence of TFA (Fig. 1A), HCl (Fig. 1B), and heptafluorobutyric acid (HFBA, Fig. 1C). Dilute HCl (6 mM) maintained suppression of the carboxyl groups without the ion-pairing effect seen with TFA, leading to reduced retention of peptides. In contrast, 6 mM HFBA formed a bulky ion pair leading to increased retention of peptides when compared to TFA. Comparison of the HCl (Fig. 1B) chromatogram to TFA (Fig. 1A) indicated the following selectivity changes: (a) region 1 had greater resolution of the more hydrophilic peptides, (b) region 2 resolved an additional peak, (c) region 3 had peak rearrangement, and (d) peak 4 had altered mobility when compared to adjacent peaks. With HFBA (Fig. 1C), all peptides were retained longer with significant selectivity changes seen with the more hydrophilic peptides. The elution of the peaks in regions 3 and 4 was retarded and the elution order of the last two peptides, region 5, was inverted. Also, each modifier had important spectral characteristics. With HFBA, there was a large baseline shift due to
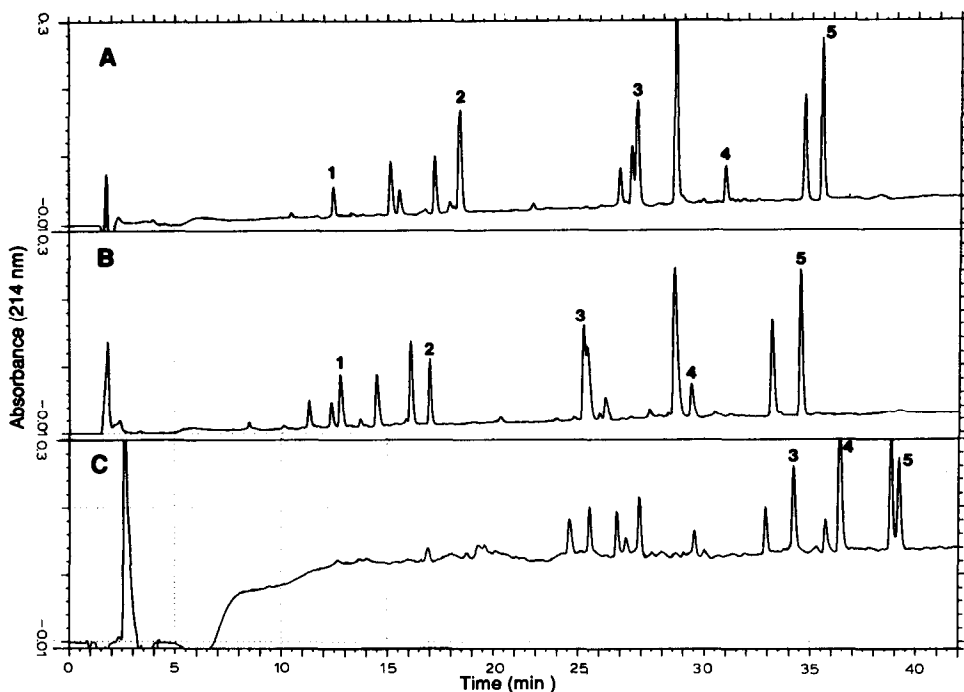
Fig. 1. Effect of mobile phase modifier on reversed-phase separation of tryptic peptides. The selective effects of ion-pair reagents were tested by separating a tryptic digest of chicken cytochrome c (1.3 nmol) on a non-metallic Delta-Pak C_{18} column (150 × 3.9 mm) at 35°C with a flow-rate of 1 ml/min with eluent A = water and eluent B = acetonitrile each containing the same concentration of modifier with a linear gradient (0–60% B, 33 column volumes) in the presence of 0.1% TFA (A), 6 mM HCl (B), and 6 mM heptafluorobutyric acid (C).

the increased extinction coefficient of this acid. Conversely, the baseline shift was diminished with HCl. The relative optical clarity of HCl may facilitate detailed spectral characterization in the low UV where the side chains of non-aromatic amino acids absorb.

Temperature
    Peptide mapping at increased temperature led to reduced retention for all peptides (Fig. 2). While there were changes in selectivity, the differences were not as striking as those seen with alternative mobile phase modifiers. At 75°C (Fig. 2B), peaks 3 and 4 eluted closer to peak 5 and farther from peak 2. Also, at higher temperature, the relative distance between peak 6 and peak 7 decreased. In the chromatogram at 30°C (Fig. 2A), peak 1 was a co-elution of two peptides that differ by one Lys residue. At 75°C, peak 1 resolves the two components. However, due to decreased column life, continued operation at 75°C is not recommended.

Inverted ion suppression
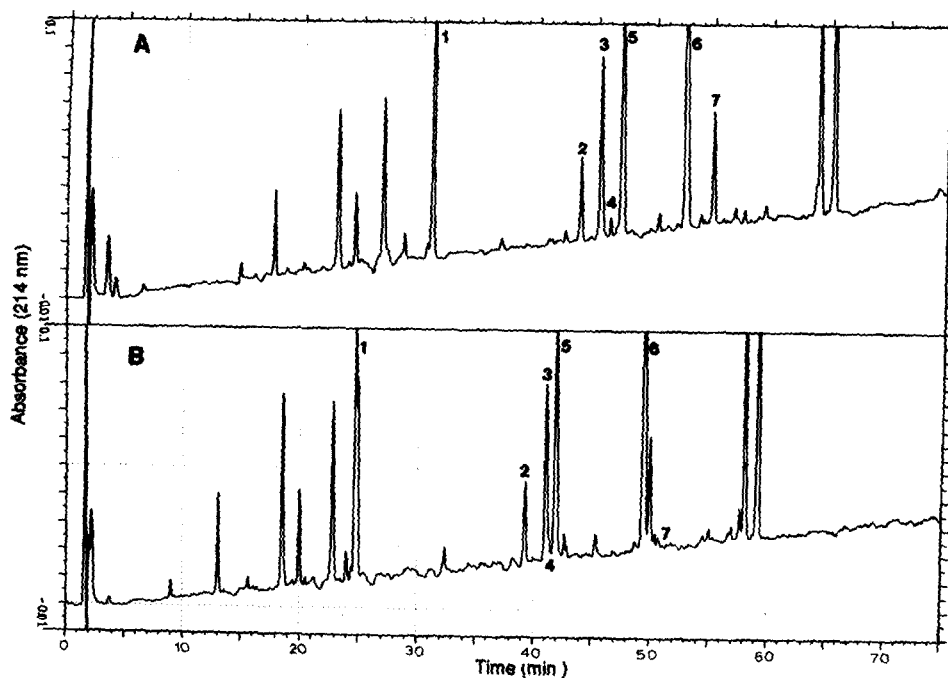   Peptide mapping is most commonly performed on silica-based packing materials

Fig. 2. Effect of temperature on reversed-phase separation of tryptic peptides. A tryptic digest of chicken cytochrome c (1.3 nmol) was separated on a stainless-steel Delta-Pak C_{18} column (150 × 3.9 mm) at 30°C (A) and 75°C (B). Chromatographic conditions: eluent A = water–0.1% TFA and eluent B = acetonitrile–0.1% TFA with a flow-rate of 1 ml/min using a linear gradient (0–60% B, 33 column volumes).

having alkyl-bonded phases where chromatography is restricted to a pH range of 2–8. The advent of polymeric or resin-based supports with extended pH ranges has increased the repertoire of the peptide chemist. The Waters RS-Pak DS-613 column performed similarly to Delta-Pak C_{18} when used with a conventional TFA gradient (Fig. 3A). While the maps were comparable (see Fig. 2A), there were marked changes in selectivity. In addition, the RS-Pak DS-613 was used in an inverted ion-pair mode, where the separation was performed at elevated pH (Fig. 3B). This resulted in ionization of the carboxyl groups and suppression of the amine functionalities. The ionized carboxyl group was, in turn, ion-paired with an alkyl amine. As seen, the selectivity was markedly altered.

Selectivity and resolution of inverted peptide mapping

Inverted ion-pair peptide maps were generated for horse, cow and chicken cytochrome c (data not shown). Many of the species differences reside in single amino acid residue substitutions that can be distinguished using this method of chromatography. Cytochrome c has a covalently bound heme group attached on tryptic peptide 14–22. In the chicken this peptide has a serine (S) residue at position 15, while in the mammalian cytochrome residue 15 is alanine (A). When the tryptic digests were chromatographed using 0.1% TFA (Fig. 4B), the heme-containing peptides could not be resolved on the basis of this single amino acid residue substitution. However, with
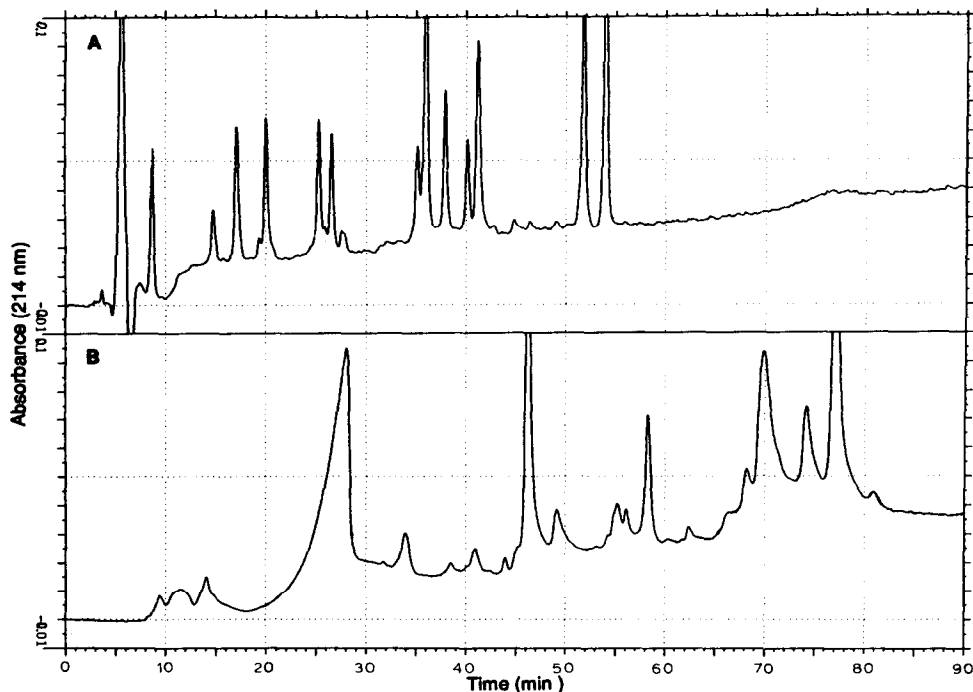
Fig. 3. Separation of tryptic peptides on polymeric reversed-phase supports. A tryptic digest of rabbit cytochrome c (2 nmol) was chromatographed at 35°C on a RS-Pak DS-613 (150 × 6 mm). For (A), the digest was separated with 0.1% TFA (pH 2.1) in water (eluent A) and acetonitrile (eluent B) at a flow-rate of 0.5 ml/min with a linear gradient (0–60% B, 14 column volumes). For (B), the digest was separated at with 2.5 mM tetrabutylammonium hydrogen sulfate (pH 11) in water (eluent A) and acetonitrile (eluent B) at a flow-rate of 0.3 ml/min with a linear gradient (10–70% B, 9 column volumes).

inverted ion-pair chromatography on the RS-Pak DS-613, these peptides were resolved (Fig. 4A). Thus, inverted ion pairing provided a distinctly different selectivity enabling resolution of the chicken peptide from the mammalian peptides.

Cation-exchange chromatography

It is possible to exploit the charge of peptides in an ion-exchange separation. Cytochrome c is a basic protein (p*I* 10), and digestion with trypsin produced several basic peptides. In Fig. 5, chicken tryptic peptides were separated on a Protein-Pak SP-5PW cation-exchange column. The basic peptides were retained on a sulfopropyl column at low ionic strength and acidic pH and separated with a salt gradient. As expected this column generated an entirely different peptide map enabling further characterization of the digest on the basis of net charge.

CONCLUSIONS

Peptide separations are most effectively improved by modification of the operating conditions and the mobile phase. Resolution is better with more shallow
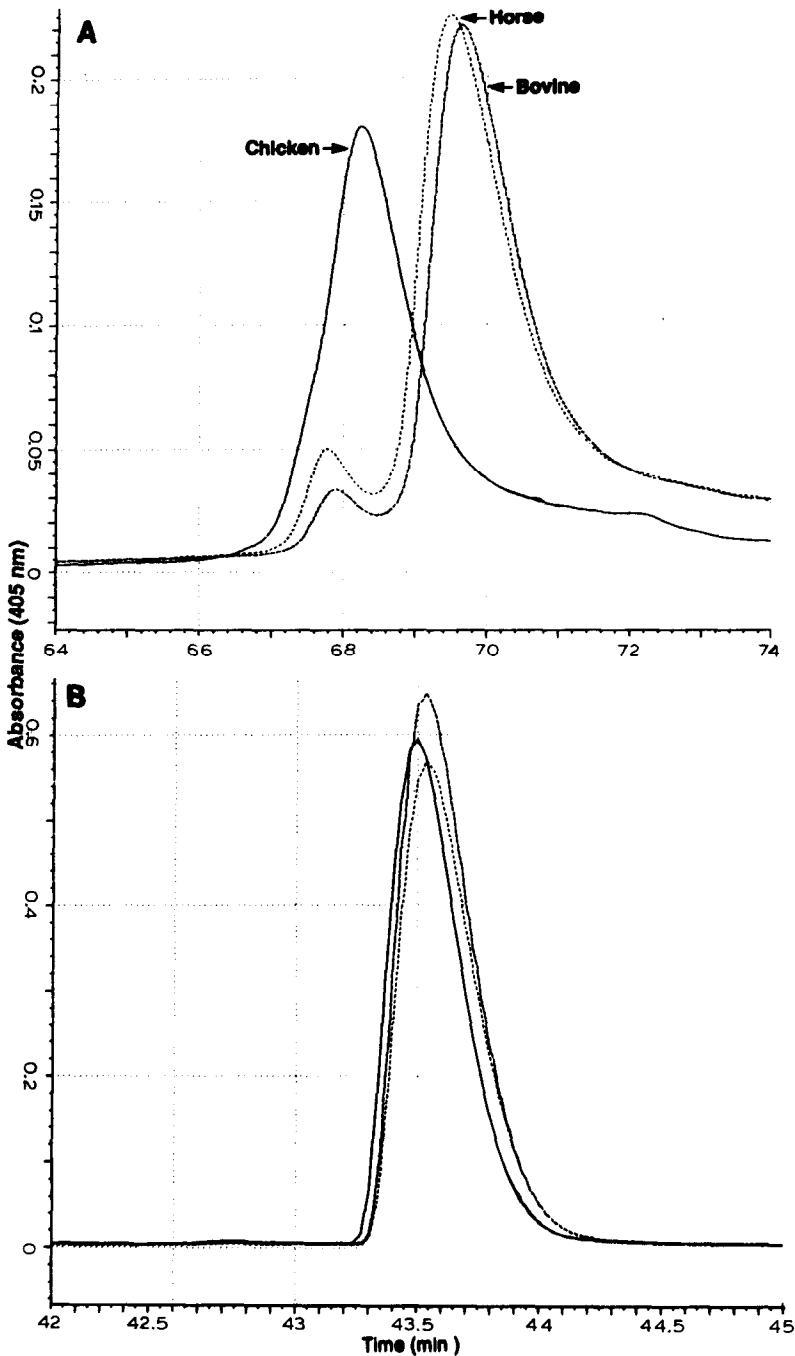
Fig. 4. Resolution in inverted peptide mapping. Tryptic digests of horse, bovine and chicken cytochrome *c* (2 nmol) were chromatographed at 35°C using inverted ion pairing (A) and standard TFA ion pairing (B). A portion of each chromatogram is shown depicting the heme containing peptides at 405 nm: chicken peptide 14–22 (CSQCHTVEK, single-letter amino acid codes) and horse/bovine peptides 14–22 (CAQCHTVEK). For (A), the digests were separated on a RS-Pak DS-613 (150 × 6 mm) with 2.5 mM tetrabutylammonium hydrogen sulfate (pH 11) in water (eluent A) and acetonitrile (eluent B) at a flow-rate of 0.3 ml/min with a linear gradient (10–70% B, 9 column volumes). For (B), the digests were separated on a Delta-Pak C_{18} (150 × 2 mm) with 0.1% TFA (pH 2.1) in water (eluent A) and acetonitrile (eluent B) at a flow-rate of 0.25 ml/min with an optimized linear gradient [0 min, A–B (95:5); 3 min, A–B (90:10); 13 min, A–B (87:13); 67 min, A–B (60:40); 77 min, A–B (40:60)].
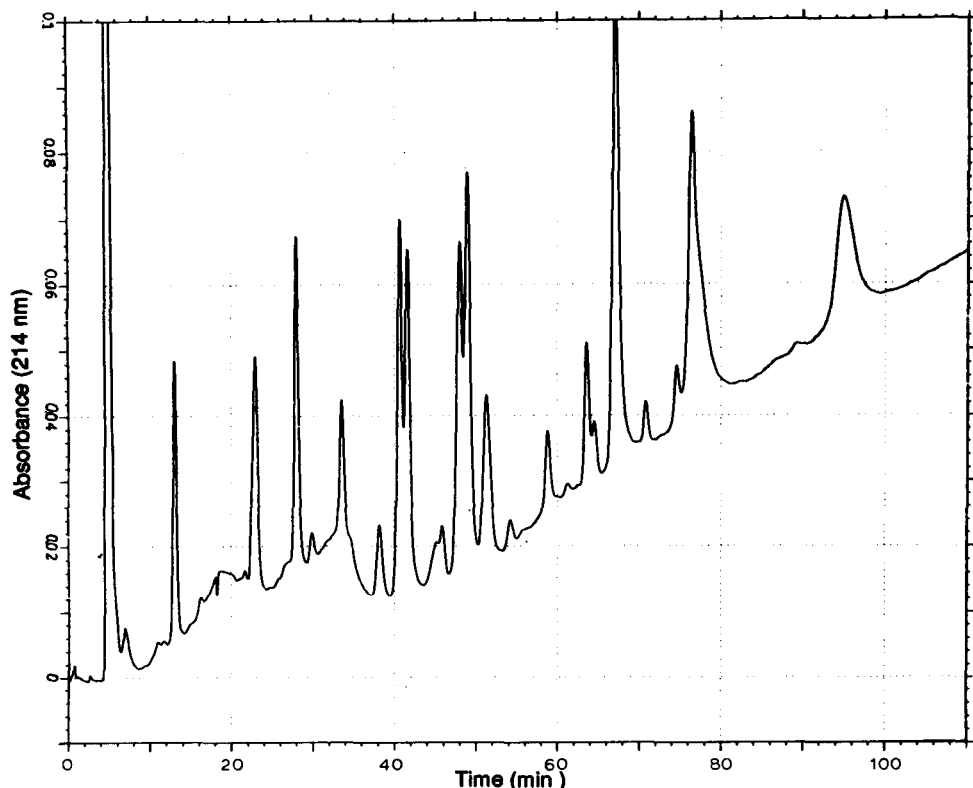
Fig. 5. Cation-exchange chromatography of cytochrome c tryptic peptides. Chicken cytochrome c (1.2 nmol) was separated on a Protein-Pak SP-5PW column at 30°C at a flow-rate of 0.5 ml/min with eluent A = aqueous HCl (6 mM) and eluent B = A + 1.0 M sodium chloride with an optimized linear gradient (0 min, 100% A; 40 min, A–B (80:20); 120 min, 100% B).

gradients. However, this approach involves a compromise between resolution and sensitivity as well as peak volume. The selectivity of the separation can be changed by small variations in pH and ion pairing associated with varying the TFA concentration. More extensive changes are observed with alternative mobile phase modifiers. HFBA increases the effect of ion pairing while only ion suppression occurs with HCl. The improved UV transparency of HCl provides enhanced opportunities for spectral characterization of peptides using photodiode array detection. The selectivity of peptide mapping is most dramatically changed by inverting the usual ion pairing and ion suppression. These options in mobile phase modification enhance the ability of the protein chemist to routinely separate all possible peptides.

Ion-exchange chromatography offers an additional mode for peptide separations. Reversed-phase chromatography relies upon ion suppression and ion pairing of the charged amino acid residues, essentially negating the charge of the peptide. For ion-exchange chromatography, it is possible to employ subtle variations of pH and ionic strength to optimize a separation based upon the net charge of the peptides. For peptide mapping, the selection of a cation or anion exchangers may depend on the pI of

the intact protein. In the case of a basic protein such as cytochrome c, tryptic fragments were separated on a strong cation-exchange column while little resolution was obtained on an anion exchanger (data not shown). Thus, the charge of the peptide becomes a major factor in determining the selectivity of the separation.

In summary, peptide mapping can be optimized on silica-based reversed-phase supports by altering the gradient slope, temperature and ion-pair reagent. Additionally, the use of polymeric supports over a wider pH range with inverted ion-pair reagents further enhances selectivity. Ion-exchange chromatography provides an additional method for peptide mapping having a dramatically different selectivity than reversed-phase techniques.

REFERENCES

1 M. T. W. Hearn and B. Grego, *J. Chromatogr.*, 266 (1983) 75.
2 M. A. Stadalius, M. A. Quarry and L. R. Snyder, *J. Chromatogr.*, 327 (1983) 93.
3 K. L. Stone and K. Williams, *J. Chromatogr.*, 359 (1986) 203.
4 J. J. L'Italien, *J. Chromatogr.*, 359 (1986) 213.
5 C. Y. Yang, E. Pauly, H. Kratzin and N. Hilschmann, *Hoppe-Seyler's Z. Physiol. Chem.*, 362 (1981) 1131.
6 E. C. Nice, M. W. Capp, N. Cooke and M. J. O'Harry, *J. Chromatogr.*, 218 (1981) 569.
7 W. C. Mahoney and M. H. Hermodson, *J. Biol. Chem.*, 255 (1980) 199.
8 H. P. J. Bennett, C. A. Browne and S. Solomon, *J. Liq. Chromatogr.*, 3 (1980) 1353.
9 J. L. Meek and Z. L. Rossett, *J. Chromatogr.*, 211 (1981) 15.
10 K. J. Wilson, A. Honegger, R. P. Stotzel and G. J. Hughes, *Biochem. J.*, 199 (1981) 31.
11 M. J. O'Hare, M. W. Capp, E. C. Nice, N. H. C. Cooke and B. G. Archer, in M. T. W. Hearn, F. E. Regnier and C. T. Wehr (Editors), *High Performance Liquid Chromatography of Proteins and Peptides*, Academic Press, Orlando, FL, 1983, p. 161.
12 W. H. Vensel, V. S. Fujita, G. E. Tarr, G. E. Margoliash and H. Kayser, *J. Chromatogr.*, 266 (1983) 491.
13 N. Takahasi, Y. Takahashi and F. W. Putnam, *J. Chromatogr.*, 266 (1983) 511.
14 W. Warren, A. Peterson, T. Wheat, M. P. Strickler, M. J. Stone, M. Dwyer and R. Pfeifer, *Am. Biotech. Lab.*, June (1989) 34.