POSTER L-704, 17th ISPPP SYMPOSIUM (Oct. 29, 1997), ROCKVILLE, MD U.S.A.

UNCHARGING A CATION-EXCHANGE COLUMN TO ELUTE PEPTIDES

Andrew J. Alpert (PolyLC Inc.,/ 9151 Rumsey Road, ste. 180/ Columbia, MD 21045 USA) [PolyLC@aol.com]

and

David A. Schooley and Kathleen M. Schegg (Dept. of Biochemistry/ U. of Nevada-Reno/ 160 Howard Med. Sci./ Reno, NV 89557-0014) ([schooley@bnr.edu] and [schegg@med.unc.edu])

Abstract

In cation-exchange HPLC, peptides and proteins are usually eluted with a gradient of increasing salt and/or pH. However, a gradient of *decreasing* pH protonates the functional groups of the stationary phase, leading to elution of retained peptides. A convenient solvent system is a gradient from 10 mM ammonium acetate to 20% acetic acid. These solvents have the advantage of being volatile but the disadvantage of absorbing below 230 nm; thus, peptides must have at least one aromatic residue in order to permit UV/VIS detection. A weak cation-exchange (WCX) material is preferable to a strong cation-exchange (SCX) material, since much less acetic acid is required to uncharge the former. Loading capacity in this mode is quite high. This method is particularly convenient for very basic synthetic peptides, which may be difficult to purify any other way, and affords good selectivity.

Introduction

Reversed-phase chromatography (RPC) is the most commonly-used general-purpose method for peptide HPLC. However, RPC fails in some cases; some peptides aren't retained and some coelute. A good alternative is cation-exchange HPLC. At pH < 4, the carboxyl- groups in peptides lose their (-) charge, and peptides have a net (+) charge. They are retained by a strong cation-exchange (SCX) materialand can be eluted by an increasing salt gradient, in order of increasing absolute number of basic residues [1,2]. This is *displacement* chromatography; the ions of the salt outcompete the peptides for the binding sites of the stationary phase. The capacity is approx. 4x greater than with RPC.

Several years ago, Michael Selsted (Univ. of Calif.-Irvine) began using PolySULFOETHYL Aspartamide™ (an SCX material) for preliminary purification of crude synthetic peptides. The peptides were adsorbed from 10 mM ammonium acetate (NH4OAc) or acetic acid (HOAc); after deprotection fragments had eluted, the peptides were eluted with a step jump to 15% HOAc. HOAc is a weak acid; it is only 1% dissociated in aqueous solution. This suggested that the mechanism of elution involved uncharging the stationary phase rather than displacement of solutes with a competing electrolyte. The volatility of the solvent made this method appealing, and we decided to study its potential in gradient elution of peptides.

Materials and Methods

HPLC apparatus and columns

The HPLC system had two LC-10A VP pumps, a SCL-10 AVP controller and a SPD-10A VP detector, all from Shimadzu Scientific Instruments (Columbia, MD).

Columns were 200x4.6 mm, packed with 5-μm, 300-Å pore material. The weak cation-exchange (WCX) material was PolyCAT A™, with a coating of polyaspartic acid covalently bonded to silica [3]. The SCX material was PolySULFOETHYL Aspartamide™, with a coating of poly(2-sulfoethyl aspartamide) bonded to silica [1].

Reagents

The phosphopeptide in Figs. 7 and 8 was synthesized with a PE-Applied Biosystems ABI 431A Peptide Synthesizer. The phosphopeptide in Figs. 4-6 and its nonphosphorylated analog (peptides E and F in the Key) were gifts of P.C. Andrews (Univ. of Michigan Medical School, Ann Arbor, MI). Other peptide standards were purchased from Sigma Chemical Co. (St. Louis, MO) or Bachem California (Torrance, CA).

Glacial HOAc and ammonium hydroxide were A.C.S. grade. NH4OAC buffers were prepared by addition of ammonium hydroxide solution to dilute aq. solutions of HOAc until the pH reached the desired level. All solutions were filtered through a 0.45 μm filter and were prepared using deionized water.

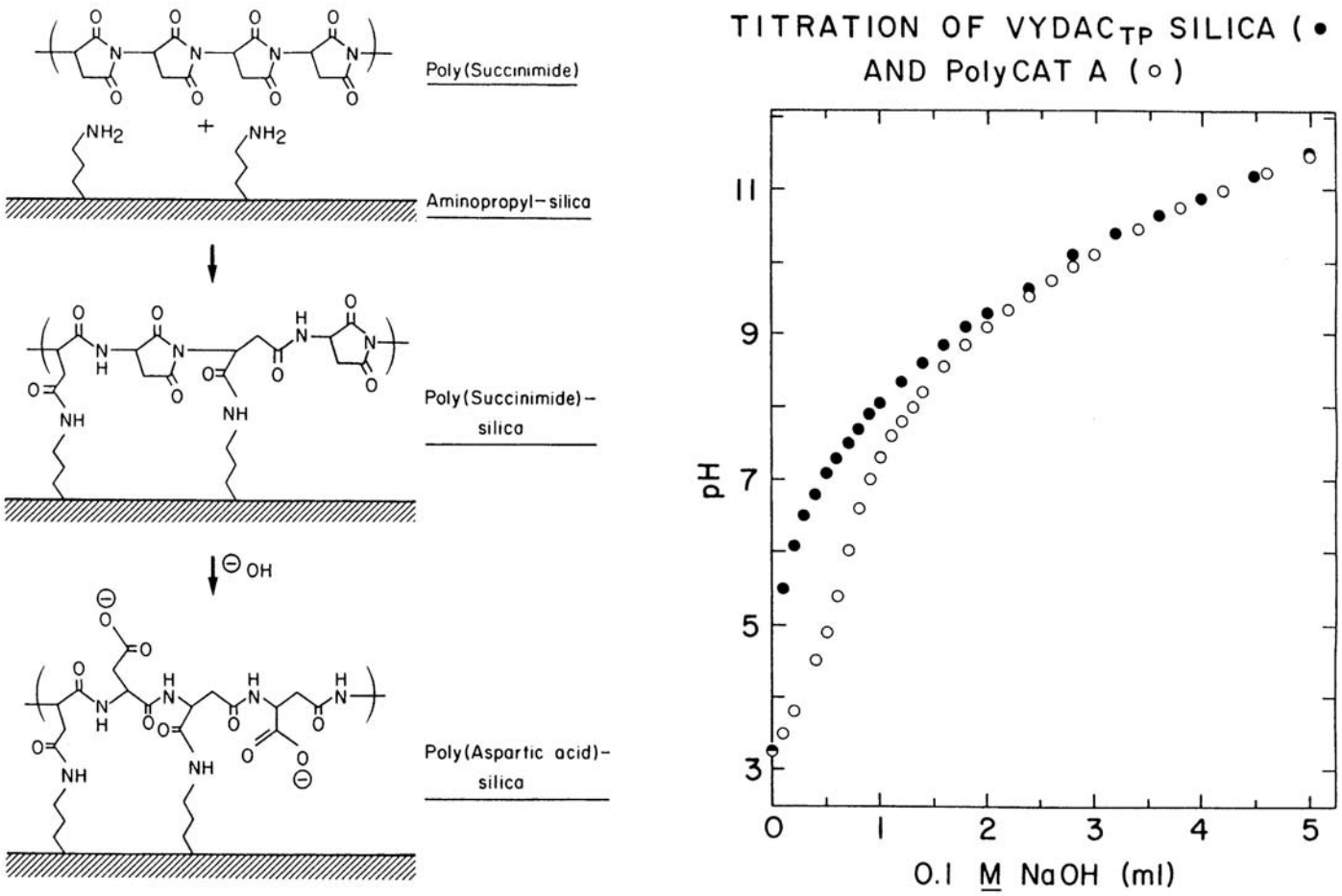

Fig. 1. Preparation of PolyCAT A.

[*LEFT*] The imide rings of the polymer polysuccinimide have a reactivity similar to that of an activated ester. They react with amino groups on the silica surface to immobilize the polymer through amide bonds. The residual succinimide rings are then hydrolyzed to yield polyaspartic acid-coated silica [3].

[*RIGHT*] The titration of PolyCAT A in suspension generates a curve characteristic of immobilized WCX functional groups.

PROTEIN STANDARDS ON PolyCAT A™

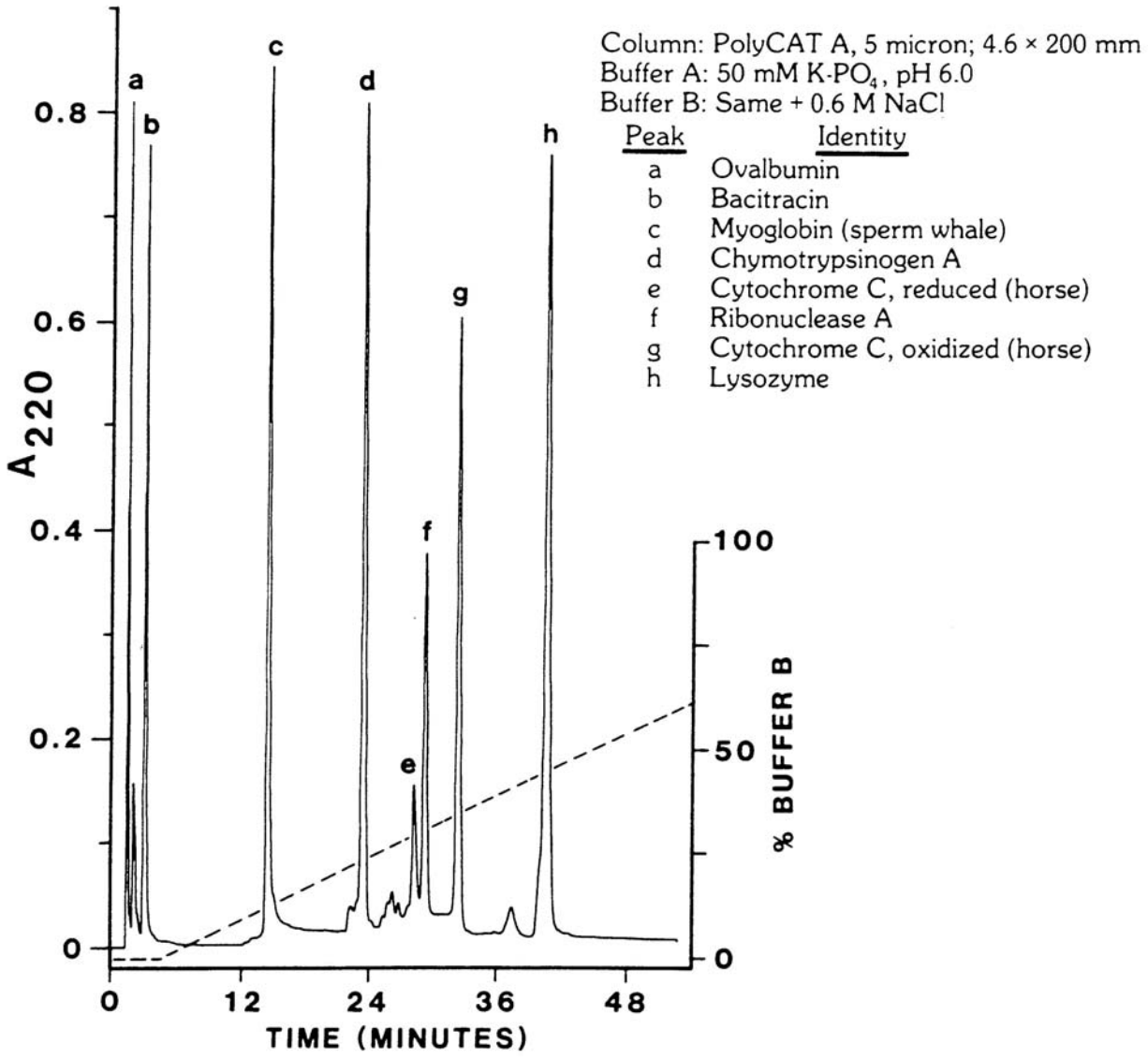

Fig. 2. Protein Standards on PolyCAT A (conventional salt elution).

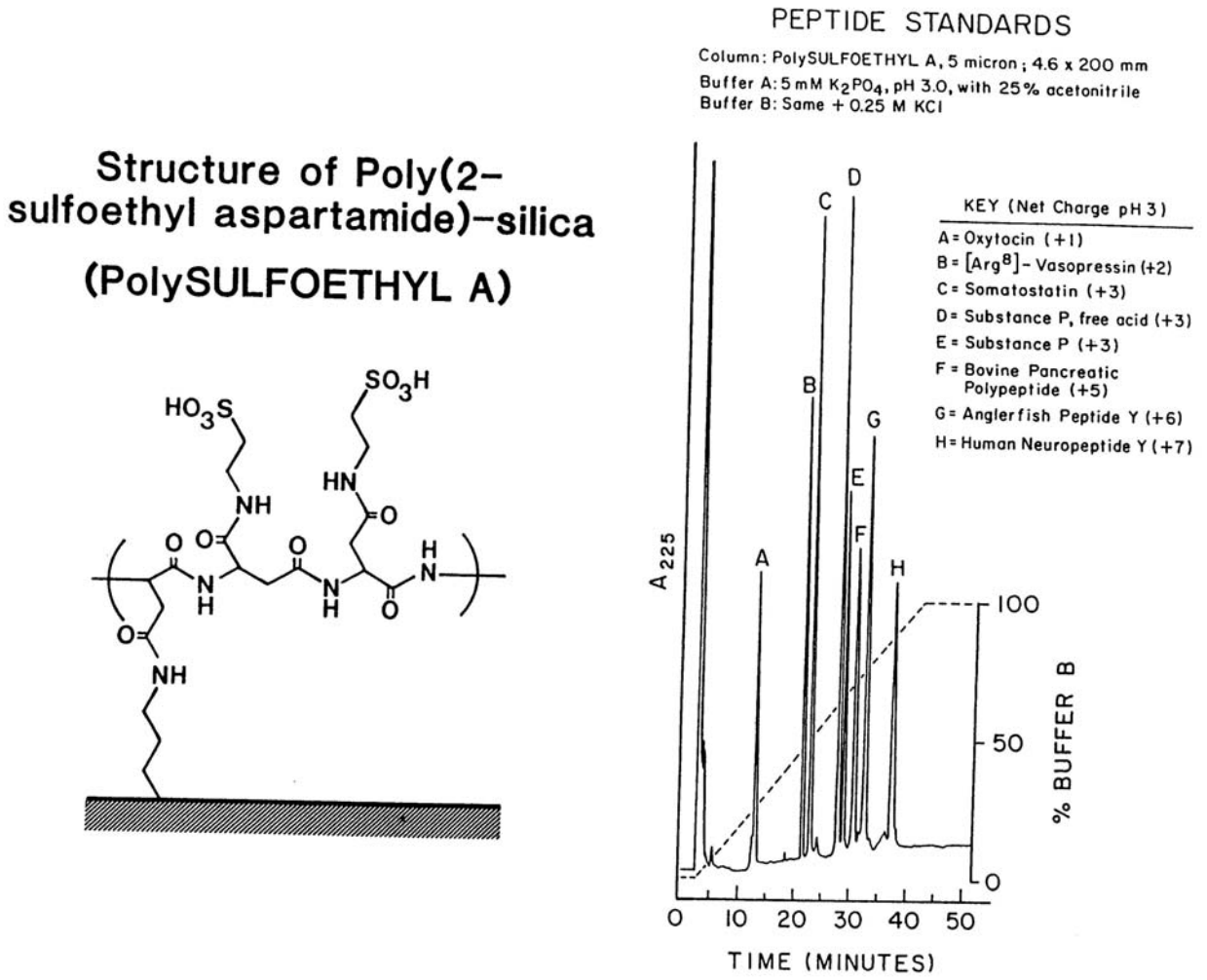

Fig. 3. Structure of PolySULFOETHYL A & Separation of Peptide Standards.

[*LEFT*] The coating is the same as with PolyCAT A, except that the amino acid taurine is amide-bonded to the carboxyl- functional groups. Sulfonic acids retain their (-) charge down to pH 2.0, the characteristic that distinguishes SCX from WCX materials.

[*RIGHT*] Elution of a mixture of peptide standards with an increasing salt gradient at pH 3.0. At this pH, the carboxyl- groups of peptides are protonated, and peptides elute in order of increasing absolute number of basic residues. The N-terminus counts as a basic residue if it's free.

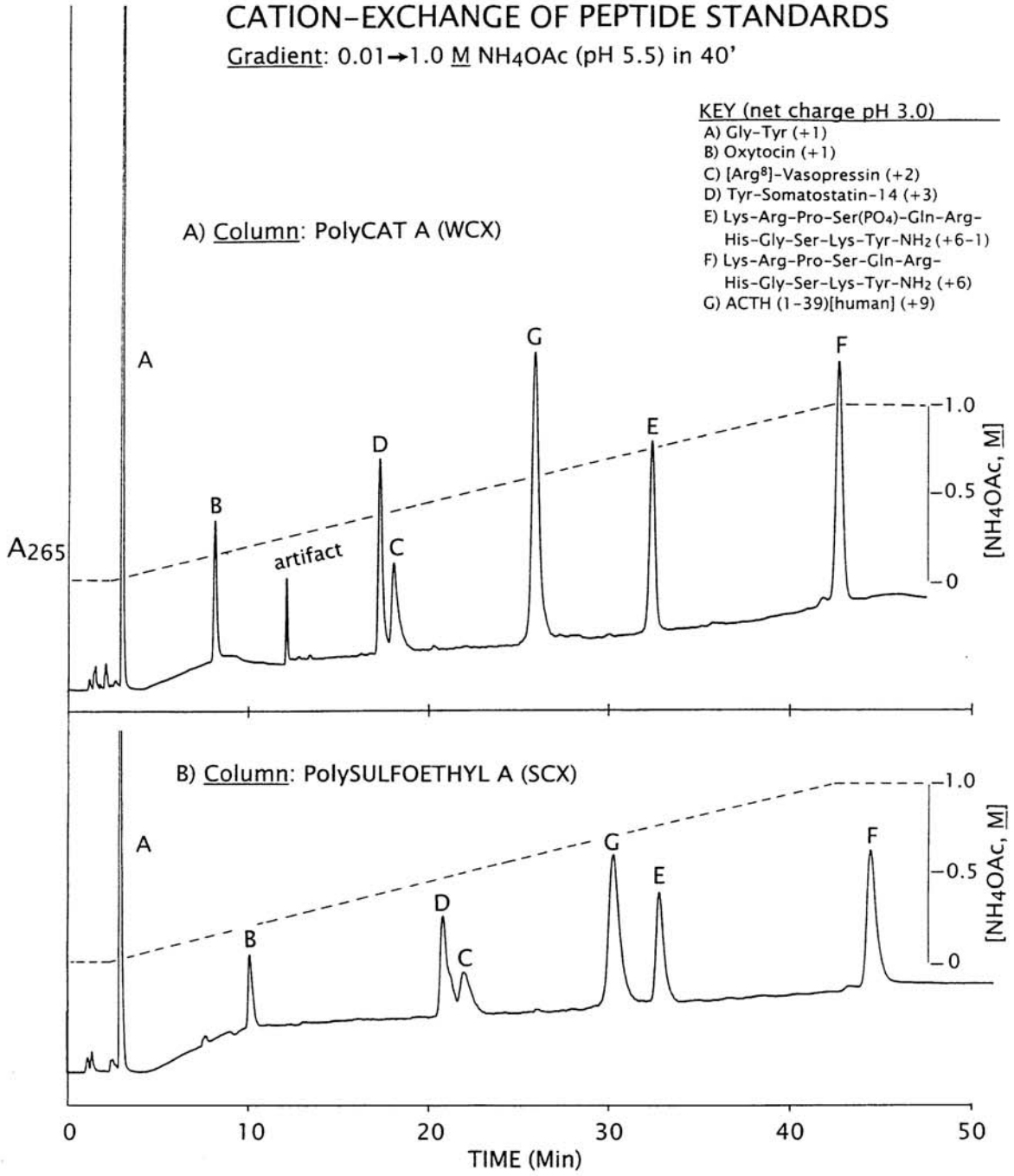

Fig. 4. Comparison of WCX and SCX materials in displacement mode.

Before comparing the materials with HOAc gradients, they were first compared with a conventional salt gradient, using ammonium acetate (NH4OAc). The peptides chosen as standards all contained a Tyr- residue to permit detection at 265 nm. Flow rate: 1.0 ml/min.

At this pH, both materials are fully charged. Under these conditions, they do not exhibit serious differences in capacity or selectivity. Also, when the carboxyl- groups in peptides are charged as well, elution isn't strictly in order of increasing number of basic residues. Peptide E elutes prior to F because of electrostatic repulsion from its phosphate group.

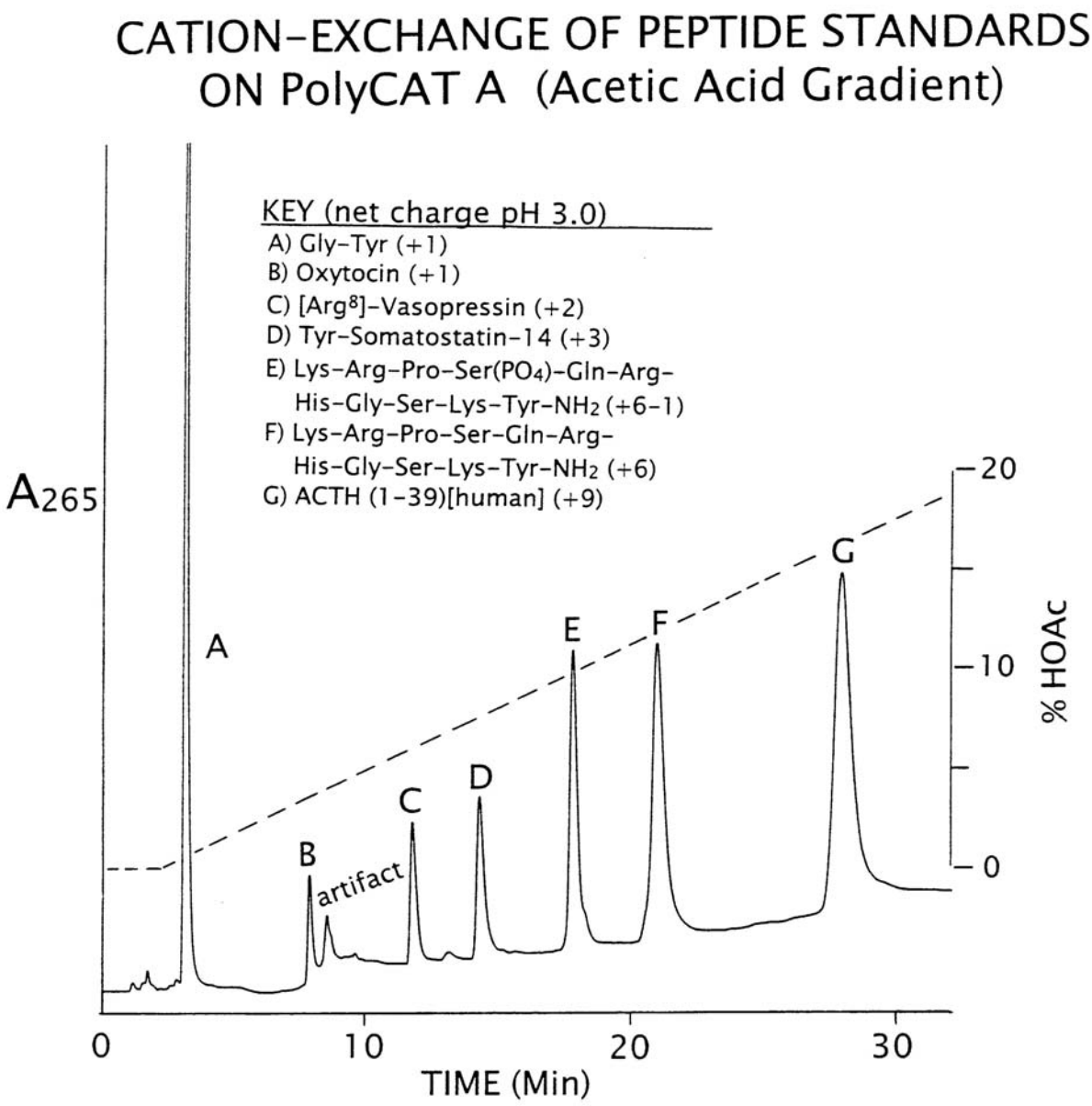

Fig. 5. Elution of Peptides from PolyCAT A with an HOAc Gradient.
Gradient: 0-100% B in 80'. A) 10 mM NH4OAc, pH 5.5. B) 50% HOAc.
Flow rate: 1.0 ml/min. A_{265} = 0.1 AUFS.

A pH of 5.5 was chosen for Mobile Phase A to insure that the stationary phase was charged initially. Under these conditions, peptides now elute in order of increasing absolute number of basic residues. 18% (3 M) HOAc suffices to elute all peptides.

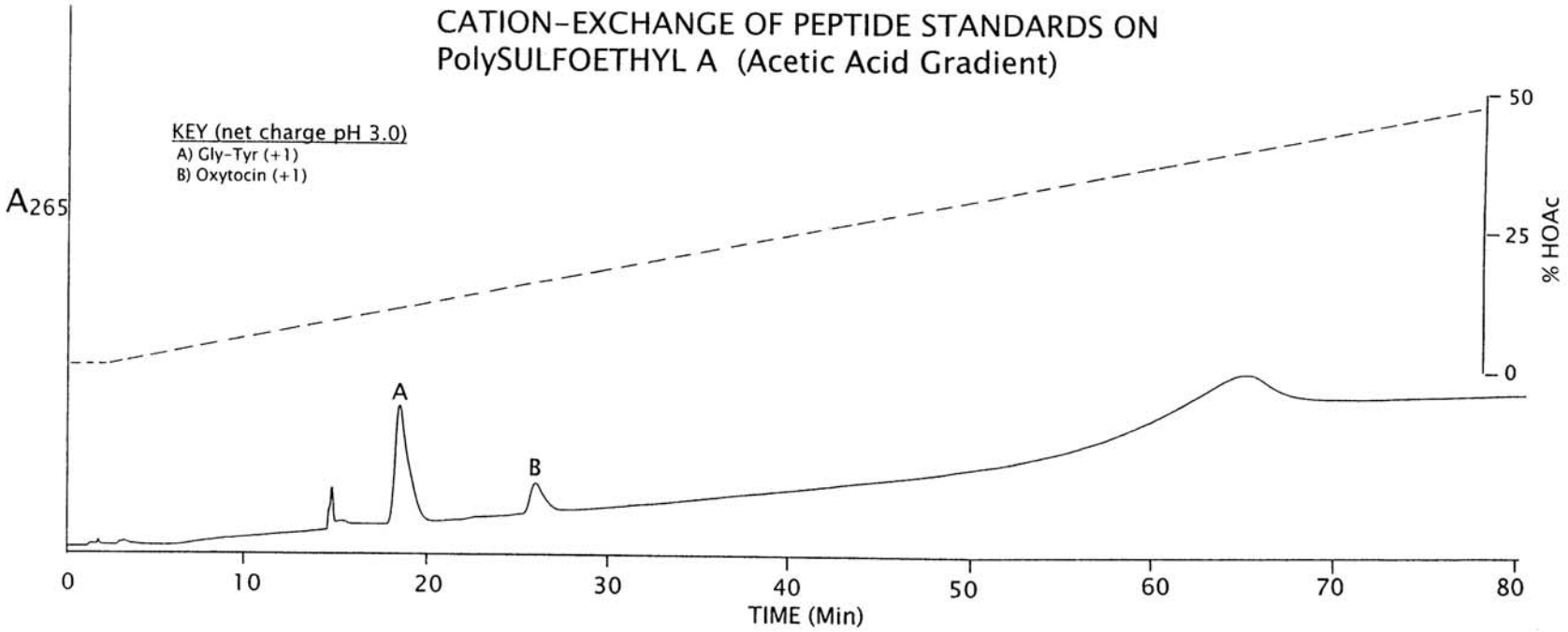

Fig. 6. Elution of Peptides from PolySULFOETHYL A with an HOAc Gradient.

Conditions: Same as in Fig. 5, but pH of Mobile Phase A = 3.5. This was chosen as a more realistic value for an SCX material.

The SCX column is much more retentive than the WCX column under these conditions; only the +1 peptides eluted in this gradient. Clearly HOAc is a much weaker eluting agent than NH4OAc (@ Fig. 4). Its success in eluting peptides from PolyCAT A establishes that the mechanism of elution is uncharging of the coating rather than displacement through competition for binding. Even 50% HOAc does not appear to uncharge PolySULFOETHYL A.

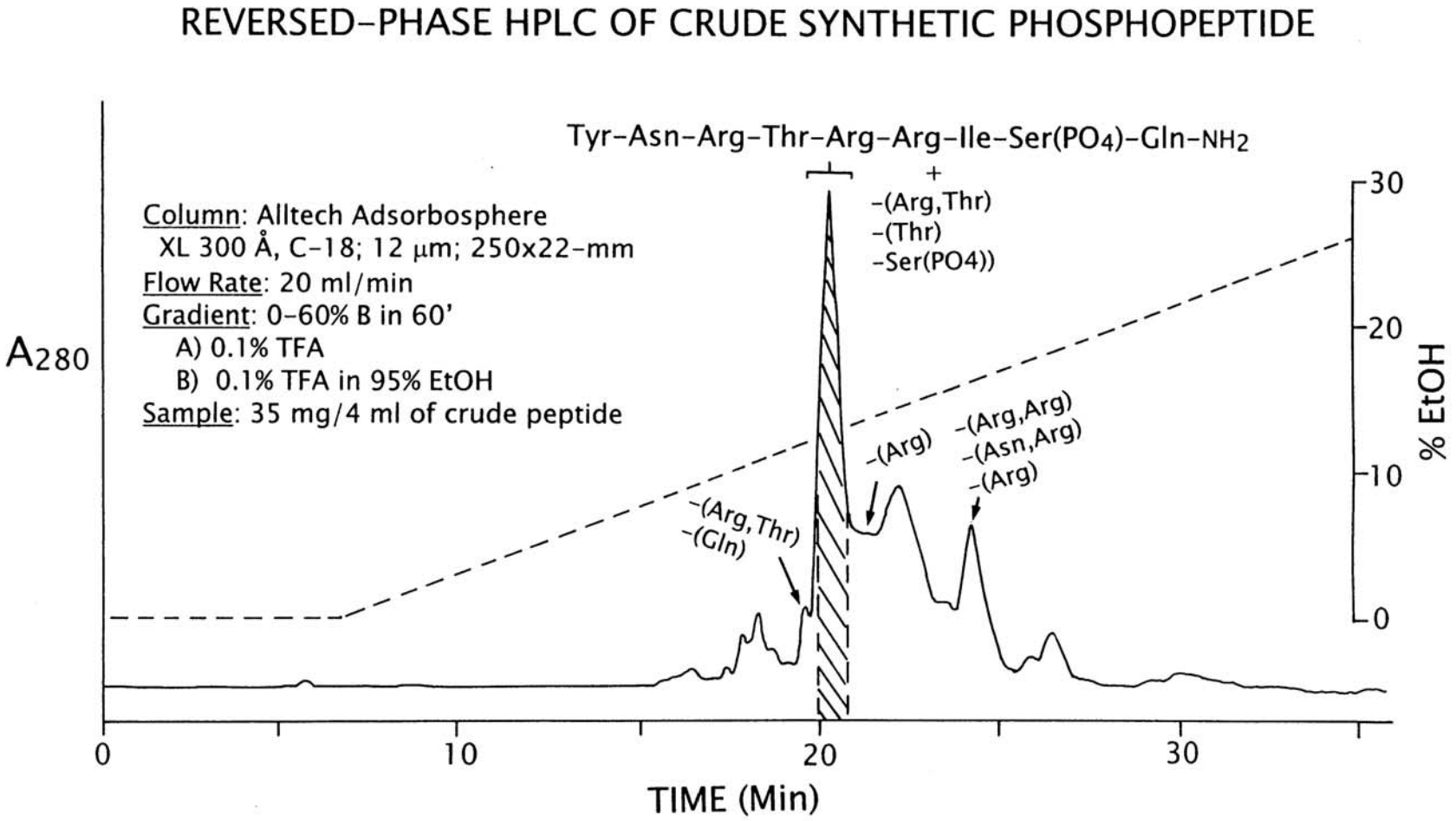

Fig. 7. RPC of Crude Synthetic Phosphopeptide.
This synthesis was attempted using a protected phosphoserine reagent. Numerous failure sequences resulted, many of which coeluted in the main peak in RPC. Accordingly, the shaded fraction was collected and repurified on PolyCAT A (Fig. 8). Some of the peaks were collected and analyzed with a Finnegan electrospray mass spectrometer. Identified deletions are shown (without specifying, say, *which* Arg- was missing, etc.).

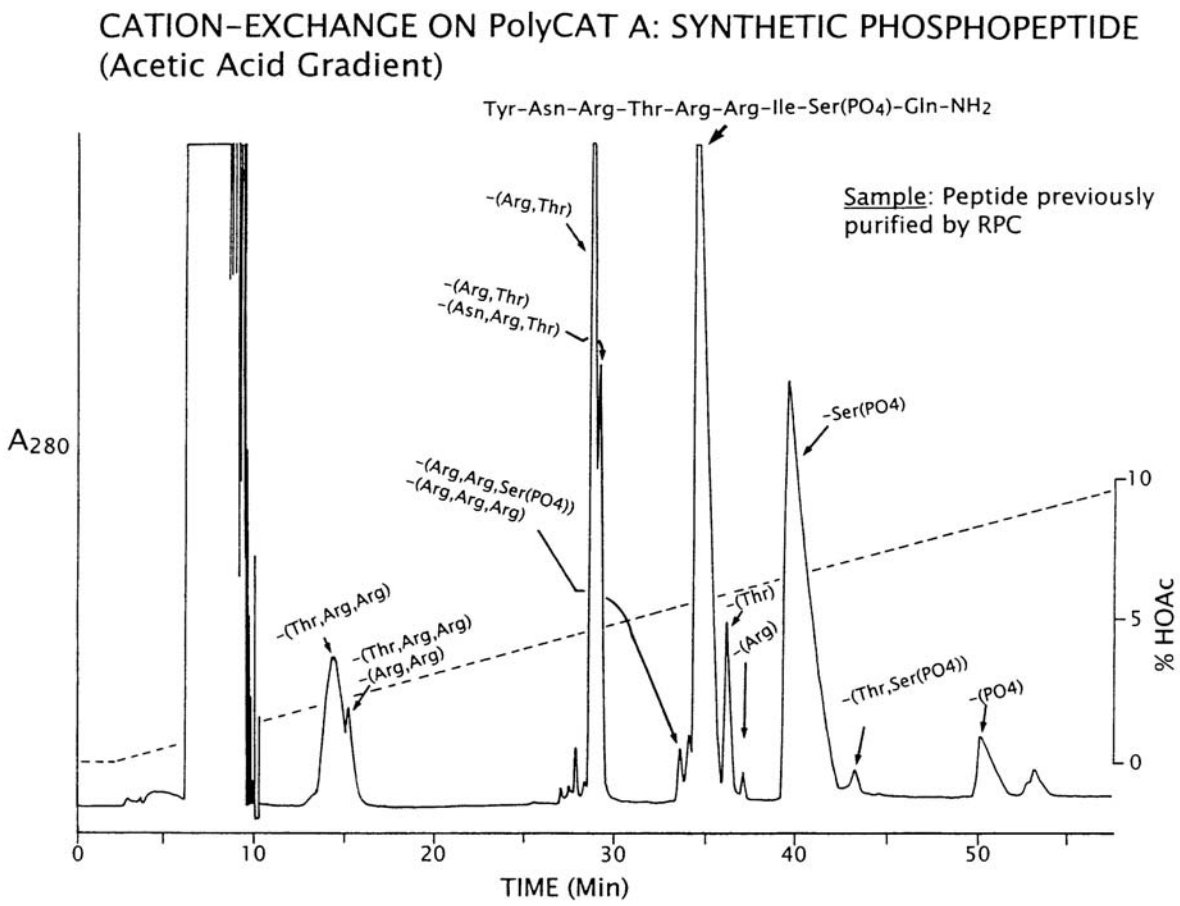

Fig. 8. Purification of Synthetic Phosphopeptide on PolyCAT A with a HOAc Gradient. Gradient: 0-25% B in 60'. A) 10 mM NH4OAc, pH 5.5. B) 40% HOAc. Flow rate: 1.0 ml/min. Detection: With these peptides, 280 nm gave better results than 265 nm.

The sample was the main fraction collected from RPC (Fig. 7). The product was purified satisfactorily at this step. Selectivity is remarkable; deletion of a Thr- residue sufficed to afford nearly baseline resolution from the main product. The elution order was as expected in some cases; loss of -PO4 or Ser(PO4) would decrease electrostatic repulsion and increase retention time, while loss of two Arg- residues decreased retention. However, loss of all three Arg- residues had little effect on retention, while loss of a single Arg- actually increased retention. With such closely-related peptides, the factors governing elution sequence are obscure.

The indicated deletions were identified by ES-MS. Again, one cannot specify which particular residue is missing if there is more than one possibility.

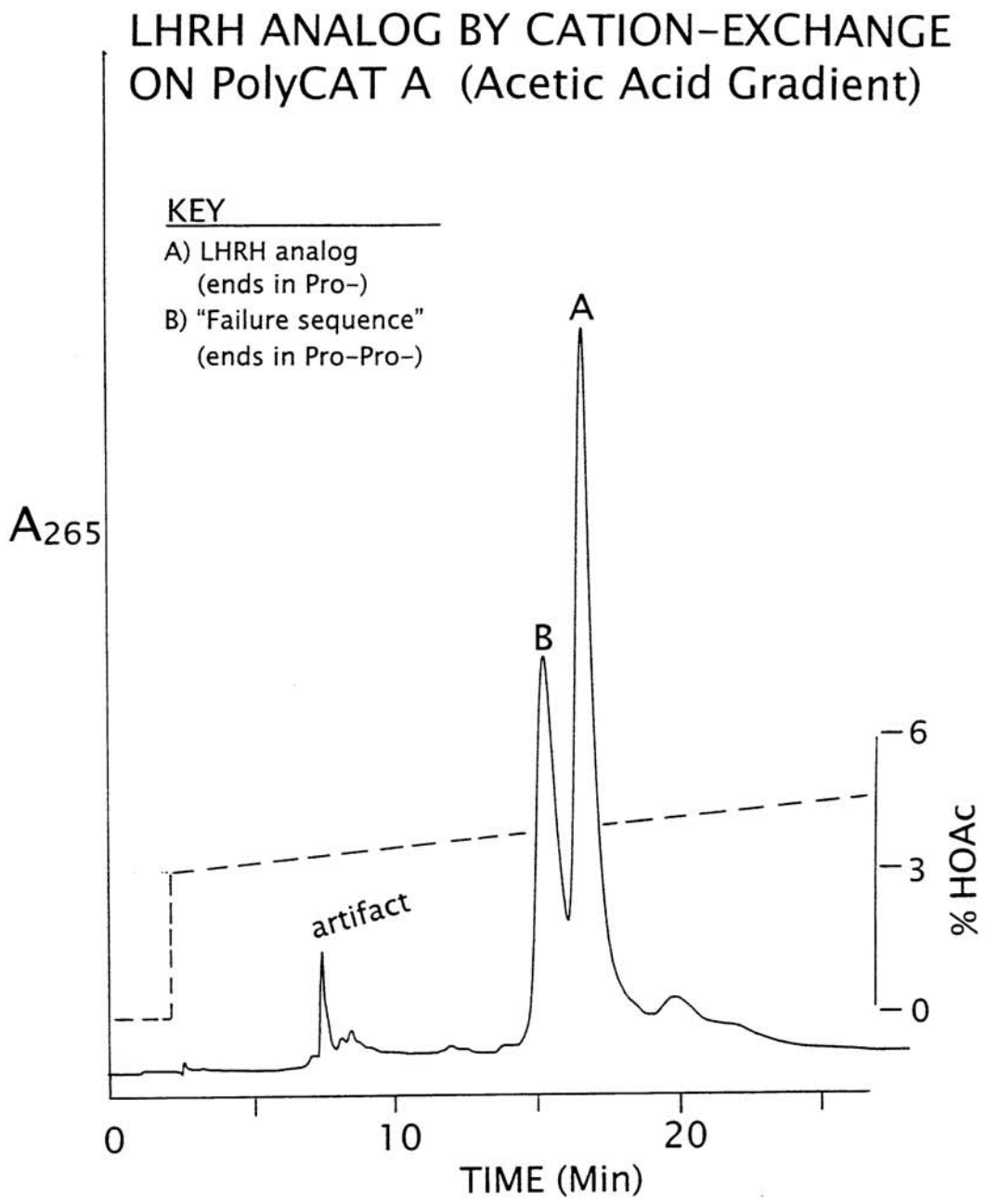

Fig. 9. Analysis of an LHRH Analog with PolyCAT A with an HOAc Gradient. Gradient: T= 0: Step jump 0-4% B, then 4-25% B in 80'. A) 10 mM NH4OAc, pH 5.0. B) 50% HOAc. Sample: Crude synthetic peptide.

This product gave a single symmetrical peak by RPC. However, ES-MS disclosed that approx. 40% of the product contained an extra Pro- at the C-terminus. Evidently there was a valve malfunction during synthesis which permitted deprotection reagent to enter the synthesis cartridge during the final addition cycle; some of the terminal Pro-residues were deprotected prematurely and accepted a second Pro-. The selectivity with PolyCAT A afforded partial resolution of these peptides, in a ratio consistent with the ES-MS analysis.

Discussion

Acetic acid is a weak acid. A 10 mM solution and a 5% solution have pH values of 3.3 and 2.6, resp. Evidently this is adequate to uncharge a WCX material such as PolyCAT A; peptides with net charges of +1 to +9 are retained and eluted with a gradient to 20% HOAc. However, HOAc is too weak an acid to uncharge an SCX material at convenient concentrations. Retention under these conditions with PolySULFOETHYL A was too strong for practical use. Stronger acids such as formic or trifluoroacetic acid might succeed in this regard, but have not been tried here.

The chief disadvantage of this method is the high absorbancy of the mobile phase below 230 nm. At the concentrations of HOAc used, its absorbancy will result in rising baselines at higher wavelengths as well, setting an upper limit to sensitivity [albeit an acceptable one]. A 50% HOAc solution has an absorbancy of 0.22, 0.03, and 0.01 AU at 254, 265 and 280 nm, resp. Several alternatives are listed on the next sheet.

The advantages of this method [next sheet] are significant, to the point that one might expect this method to have been tried before. This seems not to be the case, though. Several papers in the literature do describe the separation of basic proteins on cation-exchange columns with a gradient from 0-1 M NH4OAc in 1 M (= 6%) HOAc [4-6]. Another paper featured an NH4OAc gradient in pyridine formate [7]. These conditions would have switched the mechanism from a potentially uncharging one initially to a displacement mode during the gradient. Why has the uncharging mode not been tried before? Three factors may account for this:

1) Concern over the charge of the solute rather than the stationary phase. Elution of proteins from cation-exchange columns is promoted by an *increasing* pH gradient, which decreases the (+) charge of the proteins. Use of a decreasing pH gradient may have been counterintuitive.

2) Cation-exchange of proteins is more common than cation-exchange of peptides. This method would denature large proteins.

3) Cation-exchange is frequently performed with SCX columns, usually bearing a sulfopropyl- (SP-) functional group. Acetic acid gradients would not work well with such materials.

This technique should be useful for preparative-scale applications, owing to the high capacity, good selectivity, and inexpensive mobile phases. Peptides will be recovered in the acetate salt form, which is convenient for direct pharmaceutical applications. It will be useful for analytical purposes in cases where solutes have a reasonably high absorbancy above 230 nm. It is unlikely to be generally useful for analysis of complex protein digests, since not all of the peptides will contain an aromatic residue. Exceptions to this rule include on-line MS and other alternatives to absorbance detection.

SUMMARY: CEX WITH ACETIC ACID GRADIENTS

ADVANTAGES:

1) **General-purpose mode;** selectivity complements RPC's.
   - RPC doesn't always work; some peptides aren't retained and some coelute.
2) **Volatile solvent**; useful for mass spec, bioassays, sequencing.
3) **Prep-scale advantages**:
   - High capacity (@ 4x RPC).
   - HOAc safer to handle than TFA; cheap and easy to dispose of.
4) **Good selectivity**.
5) Peptides recovered in the acetate salt form; better for bioassays and pharmaceutical applications than the trifluoroacetate form.
6) Good solvent for peptides.
7) Easy to get very basic solutes to elute.

DISADVANTAGES:

1) **Detection**: Can't monitor < 235 nm.
   - Limited to peptides containing Phe-, Tyr- or Trp- (@ 254, 265 or 280 nm, resp.) or some other detector.
2) Denatures large proteins.
   - Not a problem if the objective is isolation for sequencing.

DETECTION WITH ACETIC ACID GRADIENTS

1) **UV absorbance**: Limited to @ 254, 265, or 280 nm for peptides with Phe-, Tyr-, and Trp-, resp.
2) **Chemiluminescent nitrogen detector (CLND)**.
   - 4x more sensitive to peptides than A_{215} but costs @ $20,000.
3) **Evaporative light scattering detector (ELSD)**.
4) **Radioactivity**.
5) **Bioassay** (@ fraction collecting).
6) **Mass spec** (@ TIC mode).

References

1) A.J. Alpert and P.C. Andrews, *J. Chromatogr. 443* (1988) 85.

2) D.L. Crimmins, J. Gorka, R.S. Thoma and B.D. Schwartz, *J. Chromatogr. 443* (1988) 63.

3) A.J. Alpert, *J. Chromatogr. 266* (1983) 23.

4) U.-H. Stenman, T. Laatikainen, K. Salminen, M.-L. Huhtala and J. Leppäluoto, *J. Chromatogr. 297* (1984) 399.

5) A.J.M. van den Eijnden-van Raaij, I. Koornneef, Th.M.J. van Oostwaard, S.W. de Laat and E.J.J. van Zoelen, *Anal. Biochem. 163* (1987) 263.

6) A.J.M. van den Eijnden-van Raaij, I. Koornneef and E.J.J. van Zoelen, *Biochem. Biophys. Res. Commun. 157* (1988)16.

7) S. Birken, G. Agosto, B. Lahiri and R. Canfield, *Thromb. Haemostas. 51* (1984)16.