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CATION-EXCHANGE CHROMATOGRAPHY OF PEPTIDES ON POLY(2-SULFOETHYL ASPARTAMIDE)-SILICA

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SUMMARY

A strong cation-exchange material, poly(2-sulfoethyl aspartamide)-silica (PolySULFOETHYL Aspartamide) was developed for purification and analysis of peptides by high-performance liquid chromatography. All peptides examined were retained at pH 3, even when the amino terminus was the only basic group. Peptides were eluted in order of increasing number of basic residues with a salt gradient. Capacity was high, as was selectivity and column efficiency.

This new column material displays modest mixed-mode effects, allowing the resolution of peptides having identical charges at a given pH. The selectivity can be manipulated by the addition of organic solvent to the mobile phases; this increases the retention of some peptides and decreases the retention of others. The retention in any given case may reflect a combination of steric factors and non-electrostatic interactions.

Selectivity was complementary to that of reversed-phase chromatography (RPC) materials. Excellent purifications were obtained by sequential use of Poly-SULFOETHYL Aspartamide and RPC columns for purification of peptides from crude tissue extracts. The new cation exchanger is quite promising as a supplement to RPC for general peptide chromatography.

INTRODUCTION

Analysis and purification of peptides has generally been accomplished in the recent past by reversed-phase chromatography (RPC). High-performance liquid chromatography (HPLC) columns of this type offer good selectivity and efficiency, and the mobile phases used are effective solvents for peptides[1]. There are a number of cases, however, where RPC is not applicable. These concern peptides that are extremely hydrophobic or hydrophilic, that tend to aggregate in RPC mobile phases, or that possess biological activity dependent upon retention of tertiary structure. In other cases, the selectivity of RPC may not suffice to resolve related peptides. Such

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separations can often be accomplished with a mode of chromatography complementary in selectivity to that of RPC. Hydrophobic interaction chromatography (HIC) is an alternative mode of peptide purification that has been used in the above situations, particularly with larger polypeptides[2–6]. However, the selectivity of HIC is too similar to that of RPC[2] for it to represent the ideal complement for general peptide HPLC.

Ion-exchange chromatography is an alternative that provides the necessary complementary selectivity, since it resolves peptides on the basis of charge rather than hydrophobicity. Ion exchange can also be used in sequence with RPC. Anion exchange has been used with acidic peptides[7] and cation exchange with basic peptides[8–11]. However, if a strong cation-exchange (SCX) column is eluted at a pH around 3, then the method becomes useful for peptides in general. This reflects the fact that Asp- and Glu- residues are uncharged at that pH, while carboxyl termini are predominantly uncharged. At the same pH, the Lys-, Arg- and His- residues and the amino termini are positively charged. Thus, any peptide with a free N-terminus will have an overall charge of at least +1 in this pH range, and should bind to an SCX column.

While a number of SCX-based peptide separations have been reported[9,12,13], the quality of the results generally does not compare well with those obtained by RPC. This may reflect the unsuitability of the base materials for peptide work, or mixed-mode effects involving the functional ligands. This report describes a new HPLC material, which has been optimized for SCX of peptides. The functional ligand is the sulfoethyl group, chosen to minimize potential mixed-mode effects stemming from the hydrophobic character of the more commonly used sulfopropyl (SP) ligand. The new material, poly(2-sulfoethyl aspartamide)-silica, features excellent selectivity and efficiency, and represents a satisfactory complement to RPC.

MATERIALS AND METHODS

HPLC apparatus and columns

The analytical-scale HPLC system was a 5500 liquid chromatograph with a VISTA 402 data system and a UV200 detector, all from Varian (Walnut Creek, CA, U.S.A.). The preparative column was run with a Delta Prep 3000 system from Waters Chromatography Division (Milford, MA, U.S.A.). Columns of poly(2-sulfoethyl aspartamide)-silica are manufactured under the tradename PolySULFOETHYL Aspartamide (formerly called PolySULFOETHYL A) by PolyLC (Columbia, MD, U.S.A.). Analytical columns have a particle diameter of 5 μm; for preparative columns particles of 15–20 μm are used. The standard pore diameter is 300 Å. The RPC column was a Vydac pH-stable reversed-phase column, 250 × 4.6 mm I.D. (The Separations Group, Hesperia, CA, U.S.A.). It was eluted with a gradient of acetonitrile in 0.1% trifluoroacetic acid (TFA) at a flow-rate of 0.7 ml/min.

Reagents

Oxytocin, [Arg^8]-vasopressin, Substance P (free acid), Neuropeptide Y (human), and β-endorphin (1–17) were purchased from Bachem (Torrance, CA, U.S.A.); β-endorphin (1–9) from Peninsula Labs. (Belmont, CA, U.S.A.); somatostatin and Substance P from Chemical Dynamics (S. Plainfield, NJ, U.S.A.); angler-

fish peptide Y (aPY) was a gift from Dr. A. Balasubramaniam (University of Cincinnati Medical Center, Cincinnati, OH, U.S.A.); and bovine pancreatic polypeptide was a gift from Dr. R. Chance (Eli Lilly Co., Indianapolis, IN, U.S.A.). The set of five retention standard decapeptides was purchased from Pierce (Rockford, IL, U.S.A.); their sequences have now been published[14,15]. All other peptides were purchased from Sigma (St. Louis, MO, U.S.A.). Reagents and solvents were of HPLC-grade or of the purest grade available.

Preparation of PolySULFOETHYL Aspartamide

PolySULFOETHYL Aspartamide was prepared by the incorporation of taurine into a coating of poly(succinimide), covalently bonded to silica, using a procedure similar to those detailed in previous papers on poly(succinimide)-based coatings[16,17].

Assay of protein cation-exchange capacity

The assay was performed with hemoglobin[16], using the recently revised value for absorbance of the standard solution[17].

Circular dichroism (CD) spectroscopy

Spectra were taken on a Model 60DS spectropolarimeter from AVIV Assoc. (Lakewood, NJ, U.S.A.). Spectra were obtained in the range 190–250 nm, at intervals of 1 nm, and represent the average of ten scans. Backgrounds were also averaged in this manner and subtracted from the spectra. Solution of *ca.* 0.5 mg/ml of the peptide in question were prepared in various solvents. A quartz sample cell with a path length of 0.1 mm was used.

Tissue extracts and protein sequence analysis

An extract of 111 g guinea pig pancreas was prepared by the method of Pettinga[18], described by Andrews and Ronner[19], except that 1.0 m*M* phenylmethanesulfonyl fluoride was present in the extraction buffers. Prior to HPLC , the dry material was dissolved in 12 ml of starting buffer. Insoluble material was sedimented by centrifugation (5000 *g*), extracted with 3 ml of saturated urea solution, and centrifuged again (5000 *g*). The supernatants were combined for injection.

Twenty-five rat posterior pituitaries were extracted with *ca.* 20 volumes of 1 *M* formic acid, containing 50 μg/ml pepstatin A. The extract was centrifuged at 5000 *g* at 4°C and the supernatant freeze-dried and resuspended in 200 μl of 1 *M* acetic acid containing 50 μg/ml pepstatin A. Insoluble material was again removed by centrifugation at 5000 *g* and the supernatant freeze-dried. The dry mixture was dissolved in 0.5 ml of starting buffer and injected into the HPLC column. Recoveries of identified peptides from the SCX chromatography of this extract were similar to those of identical extracts on RPC columns, as estimated from sequencing yields.

All peptides were sequenced on a Model 470A protein sequenator from Applied Biosystems (Foster City, CA, U.S.A.), using standard operating procedures. Peptides isolated from the PolySULFOETHYL Aspartamide columns which were sequenced were not desalted first.

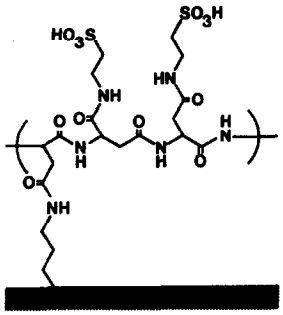


Fig. 1. Structure of poly(2-sulfoethyl aspartamide)-silica.

RESULTS

Structure and bulk properties

Fig. 1 shows the structure of the poly(2-sulfoethyl aspartamide)-silica coating. Poly(succinimide) is covalently bonded to silica, and taurine is incorporated into the resulting coating. Like other coatings derived from poly(succinimide), the coating contains both α- and β-aspartamide units. Such coatings, themselves polypeptide in nature, have proved to be broadly compatible with proteins[16,17]. The static cation-exchange binding/release capacity assay with hemoglobin (Hb) is in the range of 100 mg Hb/g packing material. This is essentially the same as that obtained with a weak cation-exchange material of a similar nature[16].

HPLC performance

Peptides are eluted in order of increasing net positive charge when a PolySULFOETHYL Aspartamide column is eluted at pH 3 with a gradient of increasing salt concentration (Fig. 2). This indicates that ion-exchange is indeed the chief mode of interaction. Peaks are quite sharp, with little tailing. The satisfactory binding of oxytocin, positively charged only at the amino terminus, suggests that the new material will be useful even for the most weakly basic peptides. Peptides with relatively high net positive charge at pH 3, such as human neuropeptide Y, are eluted with good recovery. In similar experiments, positively charged proteins, such as hen egg lysozyme and cytochrome *c*, were recovered in good yield (data not shown). Lithium chloride and sodium chloride were as effective as potassium chloride for elution of peptides.

Mixed-mode effects are evident in Fig. 2; Substance P, Substance P (free acid), and somatostatin all have the same net charge (+3) at pH 3 but are resolved to baseline. Substance P and somatostatin differ in hydrophobicity, length, and charge distribution, but Substance P and its free acid differ only in the amidation of the C-terminus. An extreme case of these effects is shown in Fig. 3. This set of five decapeptides was formulated to serve as a standard for calibration of RPC columns[20,21]; four of the peptides are identical except for variations in the hydrophobicity of two of the residues. Nevertheless, the four peptides are partially or almost completely resolved by the PolySULFOETHYL Aspartamide column (the fifth de-

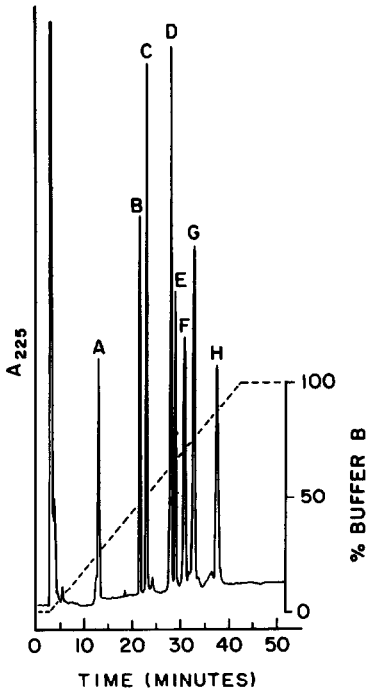


Fig. 2. Elution of peptide standards from a PolySULFOETHYL Aspartamide column (200 × 4.6 mm I.D.; 5 μm). Sample, 50 μl (initial mobile phase), containing *ca.* 5 μg of each peptide. Flow-rate, 0.7 ml/min. Buffer A: 5 m*M* potassium phosphate (pH 3.0) with 25% acetonitrile. Buffer B: as buffer A with 0.25 *M* potassium chloride. Key (net charge pH 3): A = oxytocin (+1); B = [Arg^8]-vasopressin (+2); C = somatostatin (+3); D = Substance P, free acid (+3); E = Substance P (+3); F = bovine pancreatic polypeptide (+5); G = anglerfish peptide Y (+6); H = human neuropeptide Y (+7).

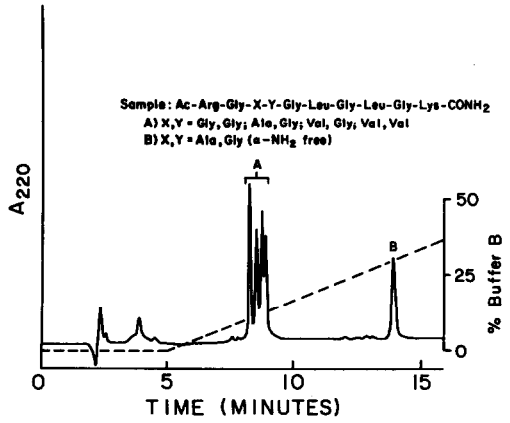


Fig. 3. Resolution of peptide standards on a PolySULFOETHYL Aspartamide column (200 × 4.6 mm I.D.; 5 μm). Sample, *ca.* 20 μg peptides in 25 μl initial mobile phase. Flow-rate, 1.0 ml/min. Buffer A: 5 m*M* potassium phosphate (pH 3.0) with 50% acetonitrile. Buffer B: as buffer A with 0.25 *M* potassium chloride.

capeptide has an extra positive charge and is eluted much later). Thus, the composition of the non-basic residues influences the selectivity to some extent.

Effect of organic solvents on selectivity

At pH 3, Substance P and its free acid form have essentially the same charge and are not resolved with aqueous mobile phases. However, addition of 25% acetonitrile to the mobile phases affords complete resolution of the pair (Fig. 4). We decided to investigate the effect on selectivity of adding varying levels of organic solvent to the mobile phases. The peptides chosen as standards were β-endorphin and some of its fragments. Some of these acquire secondary structure (α-helical regions, for example) in some solvents[22,23] and thus were promising as model peptides the retention of which might be unusually sensitive to solvent effects. Solvent effects on several other standard peptides were also examined.

Fig. 5 shows the results of this study. Increasing levels of acetonitrile in the mobile phases increase the perceived efficiency of the HPLC column. Some peptides were eluted earlier and some later. Fig. 6 depicts these trends graphically [including data for β-endorphin (1–16)]. The resolution of various peptide pairs in the mixture

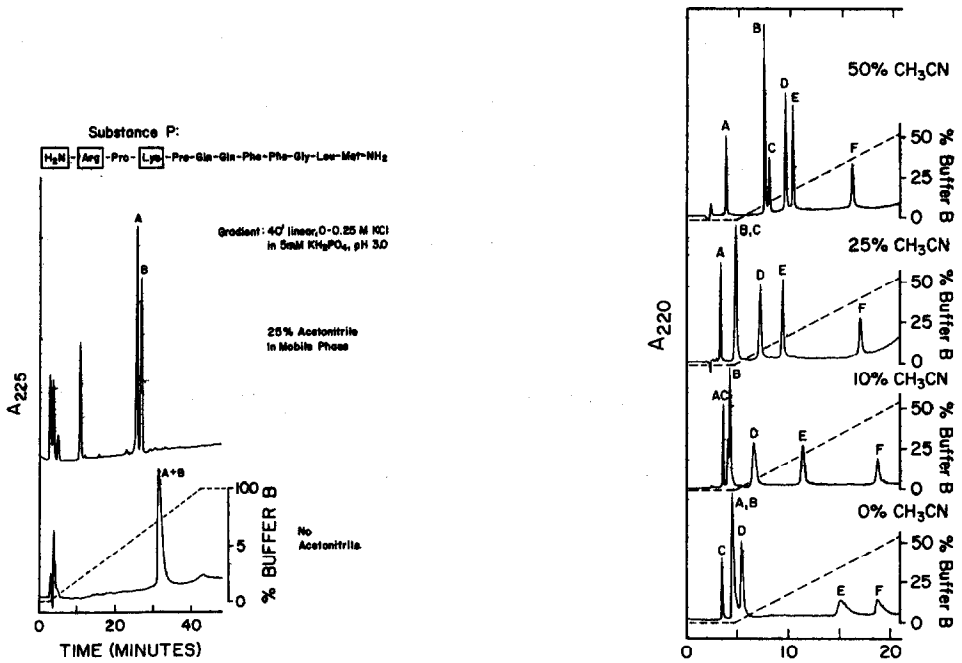


Fig. 4. Resolution of Substance P (B) and its free acid (A). For conditions see Fig. 2.

Fig. 5. Resolution of β-endorphin fragments and other peptide standards. Sample, 25 μl containing *ca.* 4 μg of each peptide; flow-rate, 1.0 ml/min. Column: PolySULFOETHYL Aspartamide (200 × 4.6 mm I.D.; 5 μm). Buffer A: 5 m*M* potassium phosphate (pH 3.0) with acetonitrile as indicated. Buffer B: as buffer A with 0.25 *M* potassium chloride. Key (net change pH 3.0): A = β-endorphin (1–5) (+1) (=[Met^5]-enkephalin; B = β-endorphin (1–17) (+2) (= γ-endorphin); C = β-endorphin (1–9) (+2); D = [Arg^8]-vasopressin (+2); E = somatostatin (+3); F = β-endorphin (+6) (human).

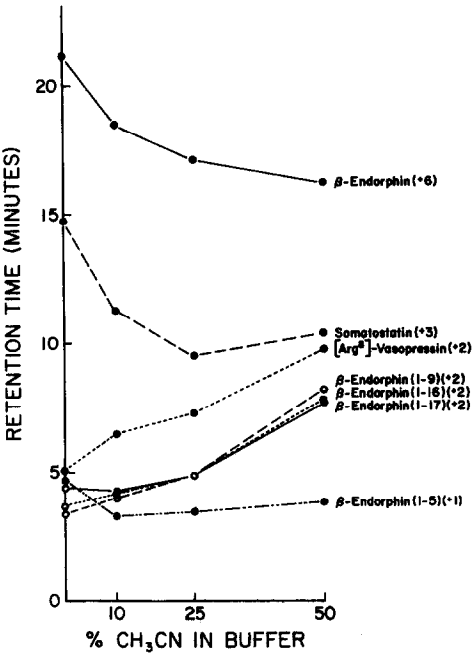


Fig. 6. Graphic representation of the data in Fig. 5, with additional data on β-endorphin (1–16).

could be increased or decreased by addition of acetonitrile, depending on the pairs involved and the level of addition.

Some retention patterns can be rationalized in individual cases. The decrease in retention of β-endorphin correlates with conditions known to shift the structure from a random coil (in aqueous solvents) to a structure with two α-helical regions[23]. Some of the six positively charged sites in the molecule could become sterically unavailable for binding to the stationary phase in this ordered structure, leading to the observed decrease in retention. β-Endorphin (1–9) is known to have a sequestered N-terminus[22]. Increasing levels of organic solvent may make this positively charged site more available for binding, leading to the observed increase in retention time.

In order to ascertain the role of structure in determining retention patterns, CD spectra were obtained for two of the peptides dissolved in the HPLC mobile phases. Osthoff *et al.*[6] have used this method to explain differences in RP and HIC resolutions of peptides. Here, β-endorphin (1–9) was selected as a peptide exhibiting increasing retention with the level of organic solvent. The sequestration of the N-terminus is explained to some extent by the patterns in Fig. 7; they are typical[24] of a β-turn structure (peptides this small with secondary structure have been previously reported[25,26]). Addition of 50% acetonitrile to the solvent does not change the spectrum markedly.

Somatostatin was chosen as an example of a peptide exhibiting decreasing retention as the level of organic solvent increases. The CD spectra of this cyclic peptide (Fig. 7) are not easy to interpret, but there is at least as much structural change with addition of 50% acetonitrile as is observed with β-endorphin (1–9). It

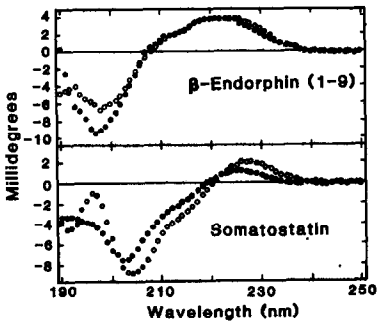


Fig. 7. Circular dichroism spectra of peptides dissolved in HPLC mobile phases. For conditions, see Materials and methods. (●) 5 mM potassium phosphate (pH 3.0); (○) the same with 50% acetonitrile.

would appear that the CD data do not permit any generalizations regarding the effect of conformational changes on peptide retention.

Chromatography in mostly organic mobile phases

Fig. 8 shows the capacity factor for the dipeptide Gly-Tyr on a PolySULFOETHYL Aspartamide column with increasing levels of methanol in the mobile phase. The capacity is higher at pH 3 than at pH 4, due to the loss of charge at the carboxyl terminus of the dipeptide. However, at levels of methanol over 60%, the two curves converge. The capacity factors increase several-fold at the same time. A similar increase in capacity was noted by Mant *et al.*[27] in connection with size-exclusion HPLC of synthetic peptides on a stationary phase with a neutral, hydrophilic coating. The authors assumed that high levels of acetonitrile promoted ionic interactions with the stationary phase. It is possible that peptides could be retained through ion pairing to stationary phases in the presence of predominantly non-aqueous mobile phases, and the stability of such ion pairs could explain the increase in capacity factors. However, the convergence of the curves in Fig. 8, and the simi-

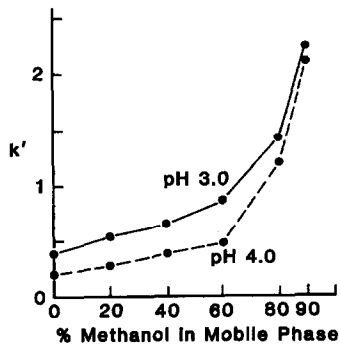


Fig. 8. Retention of Gly-Tyr as a function of the level of methanol in the mobile phase. Column, PolySULFOETHYL Aspartamide (5 μm), 200 × 4.6 mm I.D.; mobile phase (isocratic), 4 m*M* potassium phosphate, with the pH and methanol percentage indicated; flow-rate, 1.0 ml/min; sample, 5 μg Gly-Tyr in 10 μl mobile phase. The capacity factor was determined with the formula $k' = (t_R - t_0)/t_0$.

larity in behavior with a size-exclusion packing, suggest that the mode of retention in this region is no longer electrostatic. Hydrogen bonding or some other form of dipole–dipole interaction are plausible candidates for the mode of chromatography obtained with polar stationary phases and predominantly non-aqueous media. We are continuing this study to ascertain the true mechanism of retention under these conditions.

Applications to polypeptide purifications

PolySULFOETHYL Aspartamide columns should be useful for purification of polypeptides from complex mixtures, since their capacity and efficiency are high and their selectivity complements that of RPC. This was demonstrated with the purification of peptides by SCX and RPC in sequence. In Fig. 9, a crude acidic ethanol extract of guinea pig pancreas was applied to a preparative-scale column of PolySULFOETHYL Aspartamide. Indicated fractions were collected, and the components were subsequently resolved by RPC (see below), confirming that the components in the fractions were similar in charge but different in hydrophobic character.

Some of the peaks in Fig. 9 can be sequenced directly, indicating a purity in excess of 90%. This was the case with the fraction eluted at 75 min. The RPC chromatogram (Fig. 10) features a single major component the sequences of which corresponds to the amino-terminal end of prosomatostatin, not previously isolated from this source[28]. The peak in Fig. 9, eluted at 102 min, contains insulin and glucagon, as shown in Fig. 11. The peak in Fig. 9, eluted at 106 min, has a number of components (Fig. 12); the major peak, eluted at 44 min in Fig. 12, was identified as proinsulin by sequence analysis (data not shown). All peaks collected from the RPC columns which did not have blocked N-termini were successfully sequenced, and were essentially homogeneous by sequence analysis.

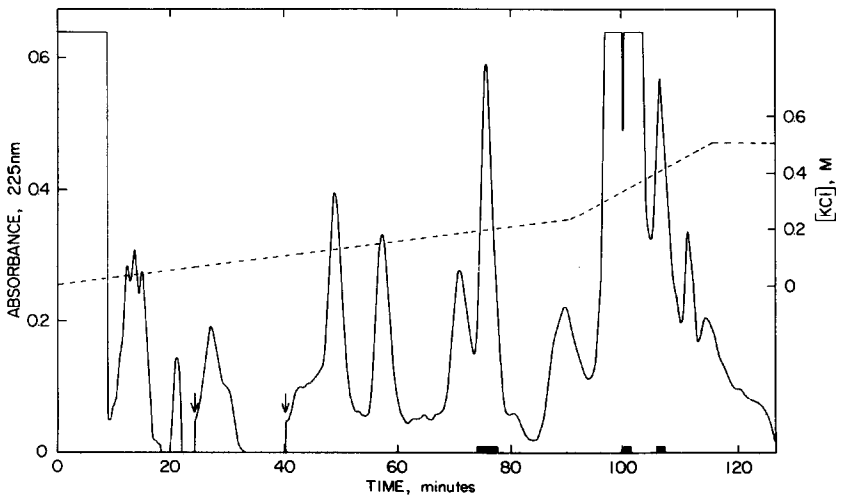
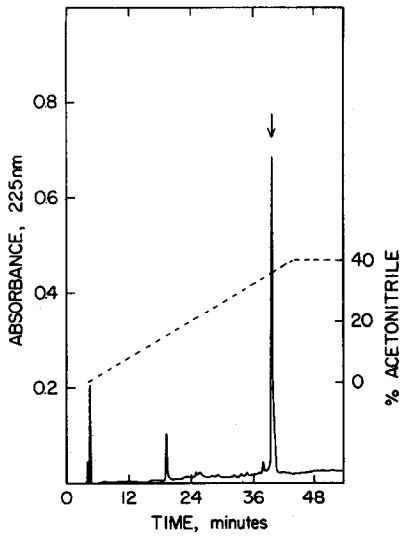


Fig. 9. Preparative purification of components from an extract of 111 g of guinea pig pancreas (see Materials and methods). Column, PolySULFOETHYL Aspartamide (15–20 μm), 500 × 22 mm I.D.; flow-rate, 30 ml/min; mobile phase, 5 m*M* potassium phosphate (pH 3.0) with 25% (v/v) acetonitrile and a gradient to 0.5 *M* potassium chloride, as shown. Solid bars indicate fractions that were subsequently rechromatographed on an RPC column (see Figs. 10–12).



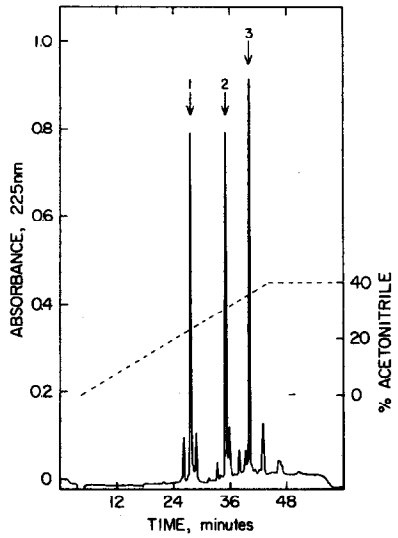
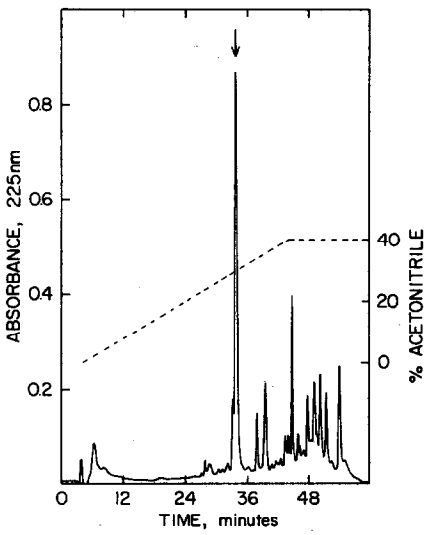


Fig. 10. RPC of the first collected fraction from Fig. 9. The major peak is the N-terminal end of prosomatostatin. For conditions, see Materials and methods.

Fig. 11. RPC of the second collected fraction from Fig. 9. Peaks: 1 = unknown (blocked N-terminus); 2 = insulin; 3 = glucagon. For conditions, see Materials and methods.



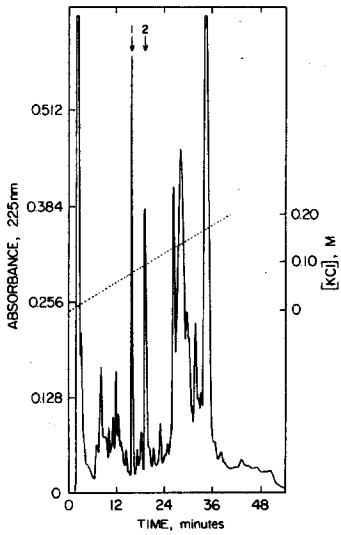


Fig. 12. RPC of the third collected fraction from Fig. 9. The second highest peak (eluted at 44 min) is proinsulin. Other peaks in this chromatogram have sequences not previously reported. For conditions, see Materials and methods.

Fig. 13. Resolution of a crude acid extract of bovine posterior pituitary on PolySULFOETHYL Aspartamide. Peaks: 1 = oxytocin; 2 = [Arg^8]-vasopressin; 3 (sharp peak at 26 min) = unknown (blocked N-terminus). Column, 200 × 4.6 mm I.D. (5 μm); mobile phase, 5 m*M* potassium phosphate (pH 3.0), containing 25% (v/v) acetonitrile and a gradient of potassium chloride, as shown; flow-rate, 1.0 ml/min; sample, 25 μl of extract (see Materials and methods).

The chromatogram in Fig. 13 demonstrates the utility of an analytical-scale column of PolySULFOETHYL Aspartamide in polypeptide purification. A crude extract of posterior pituitary was applied directly to the column. Several of the resulting peaks were collected and proved to be pure enough for identification by sequencing. Sequence analysis was performed on the peptides without desalting the collected fractions. This did not produce any apparent loss in the quality of the analyses.

Operating conditions

Silica-based column materials are quite stable in the mildly acidic mobile phases used with RolySULFOETHYL Aspartamide columns; their operational lifetime is typically in excess of 400 h. The columns work well with complex mixtures, such as crude tissue extracts or cyanogen bromide cleavage digests, but these samples tend to shorten column lifetime somewhat. Use of guard columns prevents this degradation.

We have observed no artifactual elevation in Asp-levels of peptide fractions sequenced directly after elution from PolySULFOETHYL Aspartamide columns. However, small amounts of stationary phase do tend to bleed from new HPLC columns. This could add small amounts of aspartic acid and taurine to the eluent, which would give rise to erroneous results if a peptide were to be collected for sequencing. Since most such purifications involve the sequential use of RPC and SCX, we recommend that the SCX step be performed first. The RPC step will then remove any amino acid contaminants as well as any non-lyophilizable mobile phase salts present from the SCX step. Alternatively, amino acids and salts can be removed with a sample clean-up cartridge of an RPC material prior to hydrolysis of a peptide in a collected fraction.

DISCUSSION

Chromatography with poly(2-sulfoethyl aspartamide)-silica is quite promising as a complement to RPC for peptide analysis and purification. Its efficiency is comparable to that observed with RPC, and its capacity is at least as high. Its selectivity is complementary to that of RPC and is readily manipulated by addition of varying levels of organic solvent to the mobile phases. However, optimal levels must be determined on a case-by-case basis.

Several other groups have now investigated applications of this new material. Crimmins *et al.*[29] analyzed a large number of peptides and observed retention to be in proportion to the number of basic residues; retention was also affected by oxidation, sulfation and amidation of peptides. Cheng *et al.*[30] used PolySULFOETHYL Aspartamide for the convenient isolation of the C-terminal peptides in tryptic digests; lacking Lys- and Arg- residues, such peptides are the first or among the first to be eluted. (See also Kawasaki *et al.*[31] for a thorough treatment of this method.)

Hearn[32] has observed that retention on a PolySULFOETHYL Aspartamide column is in proportion to the number of basic residues if these residues are well-separated, but that the retention effects of adjacent Arg residues are less than additive. Similar effects have been noted in other modes of chromatography; the sequence of hydrophobic residues affects retention in HIC[2] and RPC[33], for example.

The data in Fig. 6 indicate that conformational effects can also lead to the elution of a peptide at a position other than that which might be predicted from the number of basic residues. Thus, the retention time of an unknown polypeptide on a PolySULFOETHYL Aspartamide column should not be taken as a precise indication of the number of basic residues. In summary, retention of peptides on this new column material is determined predominantly by charge, but hydrogen bonding, charge distribution, conformation, and possibly hydrophobic interactions may also contribute to selectivity.

ACKNOWLEDGEMENTS

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