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HYDROPHOBIC INTERACTION CHROMATOGRAPHY OF PEPTIDES AS AN ALTERNATIVE TO REVERSED-PHASE CHROMATOGRAPHY

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SUMMARY

Hydrophobic interaction chromatography (HIC) was examined as an alternative to reversed-phase chromatography (RPC) for peptide separations by high-performance liquid chromatography. With small peptides, selectivity was similar in both modes. This was the case with commercially available standards and with a set of synthetic peptides having the same amino acid composition but different sequences. Column efficiency was higher in RPC. HIC possesses several other disadvantages, including significant baseline changes during gradient elution and a requirement for non-volatile mobile phases, which complicates peptide isolation. Thus, RPC is still the method of choice for most small peptides.

Marked differences in selectivity were noted with small proteins and polypeptides large enough to possess tertiary structure. Good results were also obtained by HIC in the case of some peptides that could not be purified at all by RPC, due to aggregation or poor binding or recovery. Thus, in these cases, HIC is a useful alternative to RPC for peptide purification.

INTRODUCTION

The purification and analysis of peptides is currently accomplished mainly by reversed-phase chromatography (RPC). High-performance liquid chromatography (HPLC) columns of this type offer good selectivity and efficiency, and are useful with many classes of peptides[1]. However, in some cases RPC is not applicable. These include peptides that tend to aggregate in RPC mobile phases (and are eluted in featureless envelopes), extremely hydrophilic or hydrophobic peptides (such as glycopeptides or integral membrane polypeptides), and peptides that lose biological activity in RPC due to loss of tertiary structure. In other cases, the selectivity of RPC may not suffice to resolve related peptides. In the above cases, it is necessary to use a mode of chromatography other than RPC.

One such alternative mode is hydrophobic interaction chromatography (HIC)[2]. To date, this method has been used almost exclusively for protein chromatography. Proteins are adsorbed to HIC sorbents from high-salt buffers; when the column is eluted with a descending salt gradient, proteins are eluted in order of

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increasing hydrophobic character at or near the surface of the tertiary structure. The elution conditions promote the retention of the tertiary conformation and the biological activity of most proteins. Thus, HIC is a suitable alternative to RPC for general protein purification.

Several recent papers indicate that HIC may be a good alternative to RPC for purification of peptides as well. Guerini and Krebs[3] found the selectivity of HIC to be superior to that of RPC for the purification of tryptic peptides from calmodulin. Ingraham *et al.*[4] subsequently used the same column in both the HIC and RPC modes with several synthetic peptides. There were marked differences in the resulting chromatograms, which correlated with conformational differences of the peptides in the different mobile phases. In view of these results, this study was undertaken to assess the utility of HIC as an alternative to RPC for general peptide HPLC. A preliminary version of this study has already been published[5].

MATERIALS AND METHODS

HPLC apparatus and columns

The 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.). HIC was performed with PolyPROPYL Aspartamide HPLC columns from PolyLC (Columbia, MD, U.S.A.). The columns were 200 × 4.6 mm I.D., with a particle diameter of 5 μm and a pore diameter of 300 Å. The preparation of the poly(propyl aspartamide) coating for the support has been described[2]. Unless otherwise noted, RPC was performed with a Vydac 218TP54 column (C_{18}), 5-μm particle diameter, 250 × 4.6 mm I.D. (The Separations Group, Hesperia, CA, U.S.A.).

Reagents

Oxytocin, [Arg^8]-vasopressin, Substance P (free acid), 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, Substance P, and [Tyr^8]-Substance P from Chemical Dynamics (S. Plainfield, NJ, U.S.A.); and β-endorphin, β-endorphin (1–16), [Met^5]-enkephalin, [Met^5]-enkephalinamide, [Tyr^1]-somatostatin, and [Tyr^{11}]-somatostatin from Sigma (St. Louis, MO, U.S.A.). The "SCDS" (same composition, different sequence) nonapeptides were synthesized by D. Lloyd of the Purdue Peptide Synthesis Lab. (Purdue University, W. Lafayette, IN, U.S.A.). The snake venom sample was prepared by L. Hsieh and F. Markland (USC School of Medicine, Los Angeles, CA, U.S.A.). The C_{18}LAP-15 peptide (a 15-residue synthetic fragment of Lipid Associating Protein, acetylated at the N-terminus with a C_{18} group) was prepared by J. T. Sparrow (Baylor College of Medicine, Houston, TX, U.S.A.). All other reagents were of HPLC-grade or of the purest grade available.

RESULTS

Synthetic peptides

Fig. 1 shows the resolution of some peptide standards by HIC. The addition or subtraction of a hydrophobic residue can change the retention time of a peptide by several column volumes. In this respect, the selectivity is similar to that obtainable

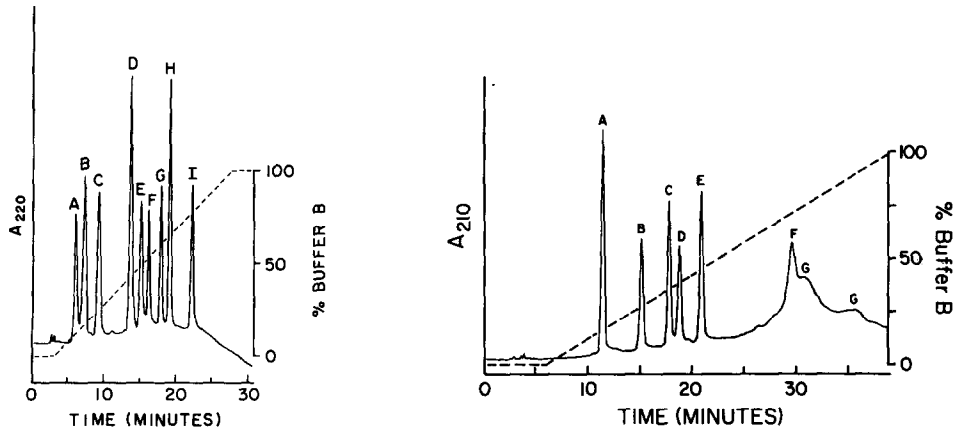

Fig. 1. Resolution of a mixture of peptide standards by HIC. Sample, 5–10 μg of each peptide in 25 μl of a 1:2 mixture of buffer A and buffer B. Buffer A, 2 *M* ammonium sulfate with 25 m*M* potassium phosphate (pH 6.5); buffer B, 25 m*M* potassium phosphate (pH 6.5). Column: PolyPROPYL Aspartamide, 5 μm, 200 × 4.6 mm I.D. Flow-rate, 1.0 ml/min; detection, A_{220} = 0.512 a.u.f.s. Key: A = Substance P (1–9); B = [Arg^8]-vasopressin; C = oxytocin; D = Substance P, free acid; E = [Tyr^8]-Substance P; F = Substance P; G = [Tyr^{11}]-somatostatin; H = somatostatin; I = [Tyr^1]-somatostatin.

Fig. 2. Resolution by HIC of β-endorphin and its fragments. Column, see Materials and methods; sample, 4–5 μg of each peptide in 25 μl of a 1:2 mixture of buffer A and buffer B. Buffer A, 2 *M* ammonium sulfate with 25 m*M* potassium phosphate (pH 6.5); buffer B, 25 m*M* potassium phosphate (pH 6.5). Flow-rate, 0.8 ml/min; detection, A_{210} = 0.512 a.u.f.s. Key: A = β-endorphin (1–9); B = β-endorphin (1–5) (= [Met^5]-enkephalin); C = β-endorphin (1–16) (= α-endorphin); D = [Met^5]-enkephalinamide; E = β-endorphin (1–17) (= γ-endorphin); F = β-endorphin (human); G = baseline artifacts.

with RPC, and correlates well with published tables quantitating the effect of amino acid composition on peptide retention in RPC[6,7]. The separation of Substance P from its free acid reflects the hydrophilic nature of the C-terminus, which is ionized at this pH. Similar behavior has been noted in RPC of peptides at neutral pH[6]. For these small peptides, then, the selectivity of HIC and RPC is comparable; the efficiency of the HIC column is somewhat less than that obtainable with a good RPC column.

Fig. 2 shows the resolution by HIC of β-endorphin and some of its fragments. Some of these peptides acquire interesting secondary structure in the presence of organic solvents and hydrophobic surfaces[8–10], and thus were promising candidates for the demonstration of selectivity differences between HIC and RPC. Nevertheless, the order of elution is essentially the same as that obtained by RPC[11,12]. The column efficiency is also lower in HIC, and there are troublesome baseline artifacts.

Sequence isomers

The retention of a peptide on a RPC column is governed mainly by its hydrophobic residues. It has been shown recently[13,14] that retention can also be influenced by the sequence of the residues. In order to compare the selectivity of HIC and RPC, a set of six nonapeptides was synthesized with the same composition but with the hydrophobic residues rearranged. Houghten and Ostresh[13] describe such a set as

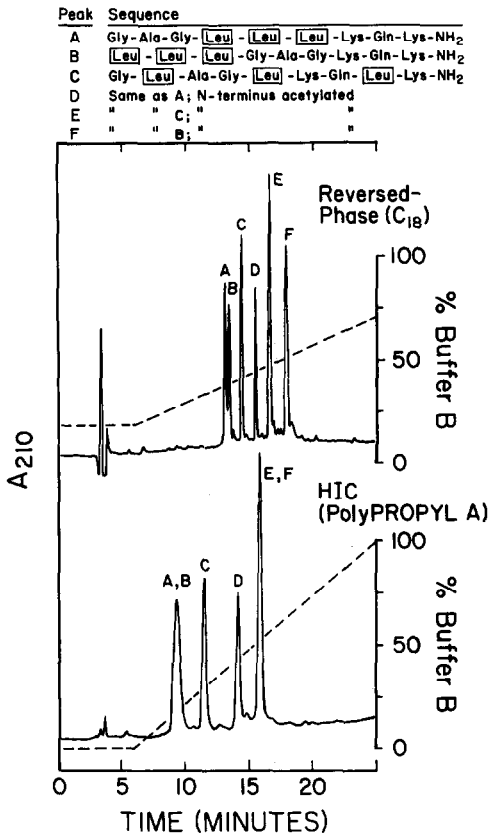

Fig. 3. Resolution of SCDS peptides by HIC and RPC. Columns, see Materials and methods. RPC: sample, 2–4 μg of each peptide in 25 μl eluent A; gradient, 30 min linear, 20–100% eluent B. Eluent A, 0.1% aqueous trifluoroacetic acid (TFA); eluent B, 0.1% TFA in 50% acetonitrile. Flow-rate, 0.8 ml/min; detection, A_{210} = 0.512 a.u.f.s. HIC: Sample, 2–4 μg of each peptide in 25 μl mixed buffer A–B (1:2); gradient, 25 min linear, 0–100% buffer B; buffers A and B, same as in Fig. 2. Flow-rate, 0.8 ml/min; detection, A_{210} = 1.028 a.u.f.s.

SCDS peptides. Fig. 3 compares the separations obtained with the two chromatographic modes. Both can distinguish between these SCDS peptides, and the selectivity is qualitatively similar. However, the selectivity of the RPC column is quantitatively superior (for this set of peptides, at least), and the efficiency is markedly better.

Application to mixtures not suited to RPC

Fig. 4 compares the separation by RPC and HIC of small proteins in a semi-purified snake venom. In this case, the selectivity of HIC is superior. The major components are unresolved in RPC but are partially resolved in HIC.

Fig. 5 shows the separation by HIC of a synthetic analogue of a lipoprotein fragment from its failure sequences and incompletely deprotected products. Attempts to purify this extremely hydrophobic substance by RPC by standard methods were unsuccessful; about 5% of the applied product was recovered, and the peaks were quite broad[15].

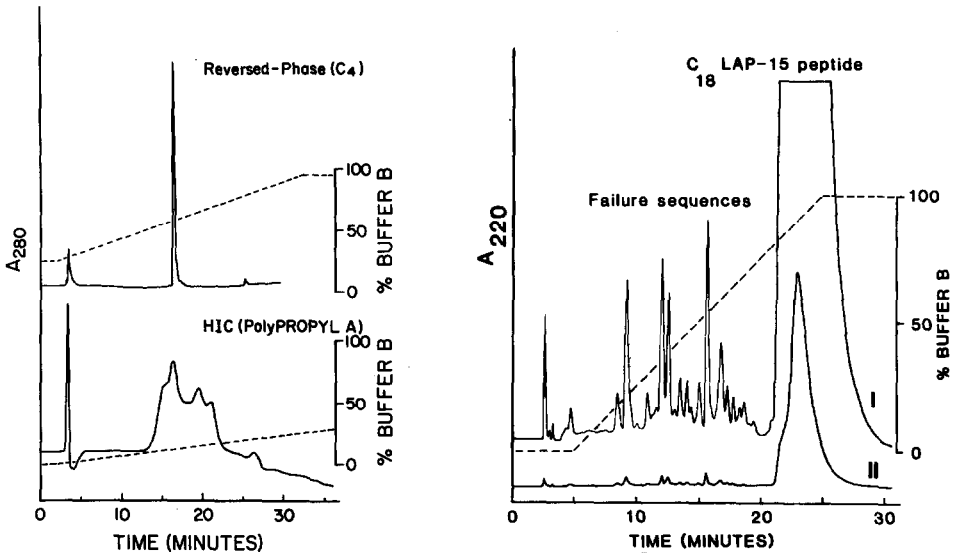

Fig. 4. Resolution by RPC and HIC of a mixture of semi-purified snake venom proteins. RPC: Column, Vydac C_4 (5 μm), 250 × 4.6 mm I.D.; gradient, 25–95% eluent B in 30 min. Eluent A, 0.1% aqueous TFA; eluent B, 0.1% TFA in acetonitrile. HIC: Column, see Materials and methods; gradient, 0–25% buffer B in 30 min. Buffer A, 1.0 *M* potassium phosphate (pH 7.0); buffer B, 50 m*M* potassium phosphate (pH 7.0).

Fig. 5. Chromatogram obtained by HIC of a synthetic LAP-15 peptide, acetylated at the N-terminus with a C_{18} group. Column, see Materials and methods; sample, 310 μg of peptide in 100 μl mixed buffer A–B (1:2). Buffer A, 2 *M* ammonium sulfate with 0.1 *M* potassium phosphate (pH 6.5); buffer B, 0.1 *M* potassium phosphate (pH 6.5). Flow-rate, 1.0 ml/min. Detection: tracing I, A_{220} = 0.064 a.u.f.s.; tracing II, A_{220} = 1.28 a.u.f.s.

DISCUSSION

HIC seems to be broadly applicable to the purification of peptides as well as proteins. The selectivity is qualitatively similar to that of RPC for peptides so small as to possess little or no tertiary structure. For such applications, RPC is to be preferred, since RPC columns tend to be more efficient. RPC also avoids such liabilities of HIC as non-lyophilizable mobile phases and severe baseline artifacts.

There are a number of separations, readily effected by HIC, for which RPC may not be suitable at all. One such class of separations is that involving quite hydrophobic peptides, as seen in Fig. 5. Another involves hydrophilic species; several mixtures of glycopeptides have been resolved on PolyPROPYL Aspartamide columns that did not bind at all to RPC columns or else aggregated in RPC mobile phases and were eluted as featureless envelopes (data not shown).

In the case of small proteins or polypeptides large enough to possess tertiary structure, the selectivities of HIC and RPC may be complementary. Fig. 4 gives one such example. Two other recent reports[16,17] also document resolutions of snake venom proteins by HIC superior to those obtained by RPC. Osthoff *et al.*[17] correlated the difference in selectivity with conformational changes evident in the circular

dichroism specta. Thus, HIC represents an alternative worth considering if RPC of larger polypeptides does not yield satisfactory results.

In cases where the two modes exhibit complementary selectivity, they could conceivably be used in sequence to purify components of complex mixtures. However, a better combination for such purposes would be RPC and cation-exchange chromatography. The latter technique has been shown to be broadly applicable to peptides[8,18], and the selectivity is more complementary to that of RPC than is the case with HIC.

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