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SYNTHETIC PEPTIDE PURIFICATION BY APPLICATION OF LINEAR SOLVENT STRENGTH GRADIENT THEORY

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SUMMARY

The purification of crude synthetic peptide by isocratic reversed-phase high-performance liquid chromatography (RP-HPLC) requires time to determine suitable chromatographic conditions, which limits the use of isocratic RP-HPLC as a purification procedure for the newer, multiple-peptide synthetic techniques. An empirical or semi-empirical procedure to determine the appropriate chromatographic conditions based on a knowledge of the peptide's sequence or molecular weight would allow direct purification of the crude synthetic peptide without the initial exploratory work. Using linear solvent strength gradient theory, a formula was derived which predicts the optimum gradient time from the number of residues in the sought-after peptide and certain chromatographic parameters. Using a variety of crude synthetic peptides of approximately twenty residues and this gradient time, the mass capacity of various commercially available RP-HPLC columns was determined. Typically, the described procedure was capable of isolating < 1 mg of purified product on a 250 mm $\times$ 10 mm I.D. column in less than 2 h. While this procedure is compatible with the small samples generated by the multiple-peptide synthetic techniques, the mass capacities are lower than has been reported previously. This is due to the definition of "optimum" in linear solvent strength gradient theory.

INTRODUCTION

Solid-phase peptide synthesis[1] has made possible the preparation of nearly any desired peptide. Furthermore, a variation of this technique developed by Houghten[2] provides for the simultaneous synthesis of large numbers of peptides, *e.g.*, the synthesis

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of 10–20 mg of each of 248 different peptides in 4 weeks. For most applications, it is necessary to purify the desired synthetic peptide from contaminating nonsense syntheses, truncated sequences and side-products. Reversed-phase high-performance liquid chromatography (RP-HPLC) is frequently used for this purpose.

The most common method of synthetic peptide purification by RP-HPLC involves the use of a shallow linear gradient in acetonitrile (*e.g.*, refs. 3–8). Rivier *et al.*[9] have published detailed guidelines for the purification of large quantities (> 100 mg) of crude synthetic peptides using RP-HPLC. Generally, however, this procedure requires a significant amount of time to develop appropriate chromatographic conditions for the preparative isolation. Further, these procedures are more suitable to larger amounts of crude synthetic peptides and are not designed to handle the 10–20 mg produced by the multiple peptide synthetic techniques.

More recently, displacement chromatography (DC) has been used for this purification problem[10]. Using DC, conventional "analytical" columns (*i.e.*, 250 mm × 4.6 mm I.D.) can be used for the purification of > 20 mg of crude synthetic peptide. While the mass sample capacity is appropriate, this method still requires preliminary work to develop a appropriate protocol, in addition to time to elute the displacer from the stationary phase prior to running the next sample.

Hodges *et al.*[11] have combined DC and gradient RP-HPLC into a novel, multi-column purification technique. As much as 89 mg of crude synthetic peptide were purified in a single run on an analytical column in approximately 2 h; thus, this technique is quite promising as a tool in routine peptide purification. However, since the report described the results for only one peptide mixture, it is difficult to assess how the selection of the exact conditions (*e.g.*, precolumn size, initial isocratic hold time, etc.) varies according to the specific nature of the crude synthetic peptide. Thus, it may prove that significant additional time is required to utilize the procedure with a wide variety of peptides.

If purification is not to be the limiting step in peptide production, the simultaneous synthesis procedure requires no more than approximately 3 h spent in purification per peptide (assuming 248 peptides synthesized in 4 weeks, with a chromatograph operating 24 h per day, 7 days per week). If an empirical formula were available for estimating chromatographic conditions which would effectively purify 10–20 mg of crude synthetic peptide in under 3 h, such a production rate would be obtainable.

One of the most successful empirical chromatographic models is the linear solvent strength gradient (LSS) theory, developed by Snyder and co-workers[12–17]. LSS theory has been used successfully to predict the chromatographic behavior of a wide variety of solutes, including polystyrene[16], peptides and proteins[17]. We have applied LSS theory to the problem of synthetic peptide purification. Using an empirical relationship derived herein between the number of amino acid residues in the desired peptide and the LSS optimum gradient volume, we have chromatographed a wide variety of peptides and examined the effects of injected sample mass on the chromatographic performance. Additionally, various commercially available columns were used, allowing a comparision of the effects of pore size, column dimensions and stationary phase. The procedure was capable of isolating < 1 mg of purified product on a 250 mm × 10 mm I.D. column in less than 2 h, without initial exploratory chromatography. While this is approximately compatible with the small samples

generated by the multiple-peptide synthetic techniques, the mass capacities are lower than has been reported previously. This is due to the definition of "optimum" in LSS theory.

EXPERIMENTAL

Peptide synthesis

The peptides used in this study were synthesized on a Applied Biosystems 430A peptide synthesizer (Foster City, CA, U.S.A.), using the recommended synthetic cycles, or manually by solid-phase techniques following generally the procedure of Merrifield[1]. The *tert.*-butyloxycarbonyl amino acids (Peninsula Labs., Belmont, CA, U.S.A.) were used with the following side-chain protecting groups: tosyl (Arg and His), benzyl ester (Asp and Glu), 2-chlorobenzyl (Lys), benzyl (Ser and Thr), formyl (Trp) and 2-bromobenzyl (Tyr). The synthesized peptides were cleaved and deprotected using anhydrous hydrogen fluoride (Matheson, East Rutherford, NJ, U.S.A.) at 0°C for 1 h with 5% (v/v) *p*-cresol and 5% (v/v) *p*-thiocresol (Aldrich, Milwaukee, WI, U.S.A.) added as scavangers. After cleavage, the resin–peptide mixture was macerated with diethyl ether (reagent grade, Mallinckrodt, Paris, KY, U.S.A.), and the crude peptide was extracted with 30% aqueous acetic acid (HPLC grade, J. T. Baker, Phillipsburg, NJ, U.S.A.).

Following lyophilization from the acetic acid solution, the crude peptides were redissolved in 0.1% trifluoroacetic acid (TFA) (HPLC grade, Pierce, Rockford, IL, U.S.A.) to exchange counter-ions. After an additional lyophilization, the crude peptide mixture was dissolved at a concentration of approximately 10 mg/ml in 0.1% TFA, filtered through a 0.45-μm membrane (Gelman Sciences, Ann Arbor, MI, U.S.A.) or centrifuged at 10000 rpm (6630 *g*) in a Microfuge 11 (Beckman Instruments, Fullerton, CA, U.S.A.), and stored at 4°C. The theoretical amino acid compositions and the sequences of the peptides used in this study are presented in Table I.

Amino acid analysis

Cleaved peptide samples were hydrolyzed in 6 *M* hydrochloric acid containing 0.1% phenol, at 110°C for 24 h. Analysis of the hydrolyzates was performed on a Beckman 6300 amino acid analyzer. The experimental amino acid compositions of the peptides used in this study are also presented in Table I.

High-performance liquid chromatography

All work was performed on a Beckman Model 344 liquid chromatograph. Detection (Beckman Model 160 UV detector) was performed at 214 or 280 nm, depending upon injected mass and peptide. Samples were injected via a syringe-loaded loop and valve. The columns used in this study, certain column characteristics and the gradient conditions are listed in Table II. In every case, a linear gradient profile was used. A Model 3390A integrator (Hewlett-Packard, Avondale, PA, U.S.A.) provided retention times, areas and peak width measurements.

Three aqueous phases were examined: 0.1% TFA; 1% triethylammonium phosphate (TEAP), pH 2.35; and 1% TEAP, pH 6.65. The latter two aqueous phases were prepared by titrating a 1% phosphoric acid solution to the desired pH with

TABLE I

EXPERIMENTAL AMINO ACID COMPOSITIONS OF THE CRUDE SYNTHETIC PEPTIDES

The analyses were performed on a Beckman 6300 amino acid analyzer. The amount of each amino acid was determined by comparison with a known amount of standard. These values were normalized to obtain the molar ratios. The values in parentheses are theoretical compositions.

Amino acid	*Experimental composition*											
	N3150[a,b]	*N4160*[a,b]	*N6180*[a,c]	*N7190*[a,c]	*N81100*[a,c]	*N101120*[a,c]	*N111130*[a,c]	*N121140*[a,b,d]	*N131149*[a,b]	*N2140*[a]	*TF1*[a]	*N14110*[a,b,d]
Asx	0.9(1.0)	0.0(0.0)	1.6(2.0)	1.2(2.0)	2.1(3.0)	1.8(2.0)	1.9(2.0)	1.1(1.0)	2.9(4.0)	2.7(3.0)	1.3(2.0)	2.6(3.0)
Thr	2.0(3.0)	1.5(2.0)	0.8(1.0)	1.0(1.0)	0.8(1.0)	1.0(1.0)	0.7(1.0)	0.0(0.0)	0.0(0.0)	1.9(2.0)	1.9(2.0)	2.1(2.0)
Ser	0.0(0.0)	0.8(1.0)	0.0(0.0)	0.0(0.0)	0.0(0.0)	0.0(0.0)	0.4(1.0)	1.0(1.0)	1.5(2.0)	0.0(0.0)	2.7(3.0)	2.5(3.0)
Glx	0.9(1.0)	3.2(3.0)	4.4(4.0)	3.2(3.0)	0.0(0.0)	1.8(2.0)	2.7(3.0)	5.1(5.0)	4.4(4.0)	1.1(1.0)	3.0(3.0)	3.0(3.0)
Gly	1.8(1.0)	2.3(2.0)	1.1(1.0)	3.4(3.0)	3.4(3.0)	1.0(1.0)	0.0(0.0)	0.0(0.0)	1.2(1.0)	1.1(1.0)	1.4(1.0)	1.4(1.0)
Ala	0.0(0.0)	2.3(2.0)	1.0(1.0)	1.1(1.0)	2.2(2.0)	3.2(3.0)	2.7(2.0)	2.2(2.0)	2.5(2.0)	0.0(0.0)	2.1(2.0)	2.2(2.0)
Cys	0.0(0.0)	0.0(0.0)	0.0(0.0)	0.0(0.0)	0.0(0.0)	0.0(0.0)	0.0(0.0)	0.0(0.0)	0.0(0.0)	0.0(0.0)	0.0(0.0)	0.0(0.0)
Val	0.8(1.0)	1.2(1.0)	2.1(2.0)	1.1(1.0)	1.3(1.0)	3.2(3.0)	2.9(2.0)	0.0(0.0)	0.0(0.0)	2.2(2.0)	0.0(0.0)	0.0(0.0)
Met	0.8(1.0)	0.0(0.0)	0.8(1.0)	0.0(0.0)	1.1(1.0)	0.0(0.0)	0.0(0.0)	0.0(0.0)	0.0(0.0)	2.0(2.0)	0.0(0.0)	0.0(0.0)
Ile	0.0(0.0)	0.0(0.0)	1.0(1.0)	0.9(1.0)	1.0(1.0)	0.0(0.0)	0.0(0.0)	1.1(1.0)	1.0(1.0)	0.0(0.0)	0.0(0.0)	0.0(0.0)
Leu	2.6(3.0)	0.0(0.0)	0.0(0.0)	1.2(1.0)	1.1(1.0)	2.0(2.0)	0.8(1.0)	2.1(2.0)	1.2(1.0)	4.7(4.0)	1.0(1.0)	1.0(1.0)
Tyr	0.0(0.0)	1.0(1.0)	0.0(0.0)	1.0(1.0)	3.1(3.0)	2.0(2.0)	1.8(2.0)	0.0(0.0)	0.0(0.0)	0.9(1.0)	0.0(0.0)	0.0(0.0)
Phe	0.8(1.0)	0.0(0.0)	1.5(2.0)	0.9(1.0)	0.0(0.0)	0.0(0.0)	0.0(0.0)	0.0(0.0)	0.0(0.0)	0.9(1.0)	0.0(0.0)	0.0(0.0)
His	0.9(1.0)	0.8(1.0)	0.0(0.0)	0.0(0.0)	0.0(0.0)	0.0(0.0)	1.2(2.0)	1.0(2.0)	0.0(0.0)	0.0(0.0)	1.0(1.0)	1.0(1.0)
Lys	3.3(3.0)	4.0(4.0)	5.5(5.0)	3.0(3.0)	2.1(2.0)	2.1(2.0)	2.3(2.0)	4.5(4.0)	3.2(3.0)	1.9(2.0)	2.1(2.0)	2.0(2.0)
Arg	0.8(1.0)	0.0(0.0)	0.0(0.0)	2.2(2.0)	1.9(2.0)	0.9(1.0)	1.6(1.0)	1.0(1.0)	0.0(0.0)	1.0(1.0)	0.0(0.0)	0.0(0.0)
Pro	4.4(3.0)	2.9(3.0)	0.0(0.0)	0.0(0.0)	0.0(0.0)	1.1(1.0)	0.9(1.0)	0.0(0.0)	0.0(0.0)	1.1(1.0)	1.0(1.0)	1.2(1.0)

[a] The amino acid sequences (single-letter symbols) are: N3150 = PMTFRLLLVDTPETKHPKKG; N4160 = TPETKHPKKGVEKYGPEASA; N6180 = FTKKMVENAKKIEVEFDKGQ; N81100 = RTDKYGRGLAYIYADGKMVN; N101120 = EALVRQGLAKVAYVYKPNNT; N111130 = VAYVYKPNNTHEQHLRKSEA; N121140 = HEQHLRKSEAQAKKEKLNIW; N131149 = QAKKEKLNIWSEDNADSGQ; N2140 = DTVKLMYKGQPMTFRLLLVD; TF1 = WSENADSGQATSTKKLHEP; N14110 = SEDNADSGQATSTKKLHEP.

[b] Used in recovery study with 1% 2-propanol in acetonitrile organic phase.

[c] Used in recovery study with acetonitrile organic phase.

[d] Recovery experiments were performed twice using this peptide.

TABLE II
COLUMNS AND GRADIENT CONDITIONS

Column No.	*Column manufacturer*[a]	*Bonded ligand*[b]	*Column length (mm)*	*Inner diameter*[c] *(mm)*	*Particle diameter (μm)*	*Particle pore size (Å)*	*Particle shape*	*Flow-rate (ml min^{-1})*	*Gradient time*[d] *(min)*	*Volume ratio*[e]	*Reduced velocity*[f]
1	Vydac	C_{18}	150	4.6	5	80	Spherical	1.0	48	1.00	1.6
2	Vydac	C_{18}	150	4.6	5	300	Spheroidal	1.0	48	1.00	2.2
3	Vydac	C_4	150	4.6	5	300	Spheroidal	1.0	48	1.00	1.6
4	Vydac	C_{18}	250	10	10	300	Spheroidal	3.4	117	1.04	2.2
5	Beckman	C_3	250	10	5	300	Spherical	5.6	65	0.97	1.9
6	Vydac	C_4	250	10	10	300	Spheroidal	3.4	117	1.04	2.2
7	Rainin	C_8	250	10	12	150	Irregular	2.8	130	0.97	2.2
8	Vydac	C_4	250	22	15–20	300	Spheroidal	7.1	480	1.87	1.7
9	Vydac	C_4	250	4.6	5	300	Spheroidal	1.0	80	1.00	1.6

[a] Beckman: Beckman Instruments, Fullerton, CA, U.S.A.; Rainin: Rainin Instrument, Woburn, MA, U.S.A.; Vydac: The Separations Group, Hesperia, CA. U.S.A.

[b] The bonded ligand is the alkyl moiety of the alkylsilane used to modify the silica support.

[c] Inner diameter of the column.

[d] In every case, the gradient profile was linear.

[e] Ratio of the gradient volume to the linear solvent strength optimum gradient volume, $V_{G,opt}$, which is $V_{G,opt} \approx 60.3r^2l$, where r and l are the column radius in cm and the column length in cm, respectively.

[f] Approximate reduced velocity, assuming a diffusion coefficient of $1.0 \cdot 10^{-7}$ cm^2 s^{-1} (see ref. 23).

triethylamine (both HPLC grade, Fisher Scientific, Fair Lawn, NJ, U.S.A.). Distilled-in-glass water was used for each aqueous phase. After mixing, the aqueous phases were vacuum-degassed for approximately 15 min prior to use. The organic phase was either pure acetonitrile or 1% 2-propanol in acetonitrile (both HPLC grade, J. T. Baker).

Mass recoveries were determined by injecting a known mass of crude peptide (typically 300 mg), collecting all the peaks, transferring the solutions to tared containers and weighing the containers after lyophilization. These experiments were performed with the 250 mm × 22 mm I.D. Vydac C_4 TP column only (Table II, column 8, The Separations Group, Hesperia, CA, U.S.A.).

Evaluation of column performance

The peak capacity (PC) was used to characterize the gradient performance and is defined as

$$\mathrm{PC} = \frac{t_G}{4\bar{\sigma}_t} \tag{1}$$

where t_G is the time over which the gradient occurs and $\bar{\sigma}_t$ is the average bandwidth, in time units, of the peaks in the sample[12]. Eqn. 1 was modified to use the area/height measurement, which was automatically calculated by the integrator. Assuming a Gaussian peak shape, the PC can be calculated by

$$\mathrm{PC} = \frac{t_G}{4\sqrt{2\pi}\ \overline{A/h}} \tag{1a}$$

where $\overline{A/h}$ is the average area/height. All peaks contributing more than 2% to the total area were used in the PC calculation. The influence of sample size on the peak capacity was typically determined by a series of eight different injected masses. All columns were compared using peptide TF1, which contained two major peaks eluting near one another.

In order to evaluate the suitability of the PC as a measure of gradient performance, the precision, reproducibility and generality of the PC for peptide samples must be established. As shown in Fig. 1, the agreement between the sets of data indicate that the PC dependency on injected mass is a reproducible and sufficiently precise characterization of the sample capacity of a gradient system. Further, for all peptides examined, the PC values decreased with increasing injected mass in the same general fashion (data not shown). Therefore, the PC values derived for any one peptide appear to be representative of the general performance of the chromatographic system, and the PC dependence on injected mass is an appropriate measure of preparative gradient performance.

Selection of gradient conditions

Stadalius *et al.*[17] established an empirical relationship between a solute's molecular weight (MW) and its solvent sensitivity parameter, S, which is fundamental to LSS theory, *i.e.*,

$$S = 0.48\mathrm{MW}^{0.44} \tag{2}$$

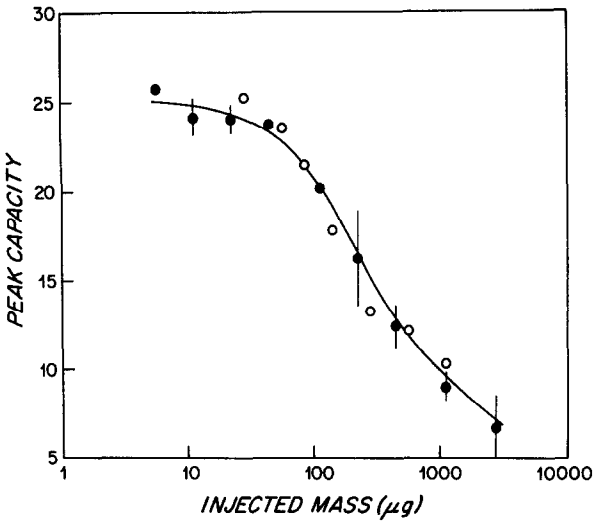

Fig. 1. Precision and reproducibility of the peak capacity *versus* injected mass. Column: 150 mm × 4.6 mm I.D. Vydac C_{18} TP column (Table II, column 2). Solvent system: 0.1% trifluoroacetic acid aqueous phase, acetonitrile organic phase; gradient of 0–50% organic in 34 min with a flow-rate of 1.4 ml min^{-1} and detection at 214 nm. The peak capacity is defined as PC $= \frac{t_G}{4\sqrt{2\pi\, A/h}}$, where t_G is the gradient time and A/h is the average area/height of the peaks. ● = Initial testing; ○ = final testing, after ~300 injections.

According to LSS theory, the optimum gradient time, $t_{G,opt}$, is given by

$$t_{G,opt} \approx \frac{\Delta\Phi S t_M}{0.3} \tag{3a},$$

where $\Delta\Phi$ is the fractional change in composition over which the gradient occurs and t_M is the column void time. Since $t_M = V_M/F$, where V_M is the empty column volume and F is the flow-rate, and, for well-packed, totally porous columns, $V_M \approx 0.8\pi r^2 l$, where r is the column radius and l is the column length, by substituting eqn. 2 and the above into eqn. 3a, we can express $t_{G,opt}$ as

$$t_{G,opt} \approx \frac{4.02\Delta\Phi MW^{0.44} r^2 l}{F} \tag{3b}$$

Further, substituting $114n$ for MW, we get

$$t_{G,opt} \approx \frac{32.3\Delta\Phi n^{0.44} r^2 l}{F} \tag{3}$$

where n is the number of residues in the peptide of interest. Eqn. 3 assumes that well packed chromatographic columns are used, and it is strictly valid only for 0.1%

TFA–acetonitrile mobile phases and octadecylsilyl-modified stationary phases. Hence, the use of this equation probably results in an overestimation of the LSS optimum volume, when applied to columns with shorter-chain alkyl ligands.

In this study, all gradients were linear and were run from 0 to 50% B (*i.e.*, $\Delta\Phi = 0.5$), which eluted even the most strongly retained peptides. A fast (*e.g.*, 10 min) 50–100% B gradient was run to wash further the stationary phase.

The flow-rates were chosen to maintain a constant product of the linear velocity and the particle size, *i.e.*, a constant reduced velocity. The use of a constant reduced velocity eliminates the shorter-diffusion-path advantage that smaller particles provide. However, this does not negate the increased plate count in a given column length, which results from using smaller particles.

LSS theory was not developed for preparative chromatography, and the optimal LSS volume represents the best compromise between time and resolution for analytical gradient separations. However, it has been demonstrated for proteins (MW 12 000–68 000) that, although operation in the mass-overloaded state results in a decreased PC relative to that in the non-overloaded state, the optimal LSS volume still provides nearly the maximum PC for a given mass load[18].

RESULTS

Mass recovery

Mass recoveries were performed gravimetrically using an injected mass of approximately 300 mg on the 250 mm × 22 mm I.D. Vydac C_4 TP column (Table II, column 8, The Separations Group). A comparison of the total mass recoveries for five peptides (Table I, footnotes *b* and *d*) chromatographed with 1% 2-propanol–acetonitrile and for five peptides (Table I, footnotes *c* and *d*) chromatographed with acetonitrile as the organic mobile phases yielded 93.8 ± 12.3% ($n = 7$) *versus* 76.8 ± 4.8% ($n = 5$), respectively. Although the variation in recovery from peptide to peptide was significant, the recovery was always higher when 2-propanol is present in the organic phase (98% confidence).

Peak capacity of TFA and TEAP phases

Fig. 2a–f shows a series of gradient chromatograms of peptide TF1, together with the injected mass and the calculated PC for each. Clearly, a PC > 20 is required to obtain resolution between the two major components in this synthetic peptide mixture in the TFA–acetonitrile system. Fig. 2g–l shows the same peptide chromatographed with the 1% TEAP, pH 2.35–acetonitrile system. At the lower loads (Fig. 2a–c and g–i), the chromatograms were nearly equivalent, with the TFA–acetonitrile system showing a greater separation for the two major peaks. However, at the higher loads (Fig. 2e, f, k and l) the sharpness of the peaks was significantly greater for the TEAP–acetonitrile system, although the two major peaks coeluted under these conditions.

Peak capacity of tested columns

To evaluate the columns, the PCs were determined for a series of separations of crude synthetic peptide TF1 (Table I) using constant reduced velocities and corresponding $t_{G,opt}$ gradient times (Table II). The resultant plots of PC value *versus*

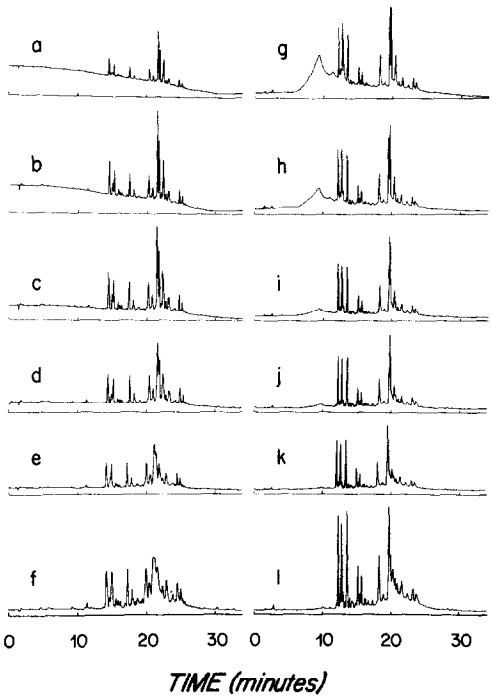

Fig. 2. Effect of the injected mass of a synthetic peptide on its gradient chromatographic separation. The peptide used is TF1 (Table I). (a–f) Solvent system: 0.1% trifluoroacetic acid aqueous phase, acetonitrile organic phase; (g–l) solvent system: 1% triethylammonium phosphate, pH 2.35 aqueous phase, acetonitrile organic phase. Other chromatographic conditions and definition of peak capacity (PC) are given in Fig. 1. Injected masses and PC values are as follows: (a) 28 μg, PC = 25.2; (b) 56 μg, PC = 23.5; (c) 140 μg, PC = 17.8; (d) 260 μg, PC = 13.2; (e) 560 μg, PC = 12.1; (f) 1120 μg, PC = 10.3; (g) 28 μg, PC = 22.7; (h) 56 μg, PC = 24.3; (i) 140 μg, PC = 24.3; (j) 260 μg, PC = 24.3; (k) 560 μg, PC = 21.7; (l) 1120 μg, PC = 16.1.

injected mass for each column are shown in Fig. 3. The maximum sample capacity was obtained on the 250 mm × 10 mm I.D. columns (Fig. 3, symbols □, ▲ and △), which provided significantly higher PC values than the 250 mm × 22 mm I.D. column (Fig. 3, symbol ◇) at moderate loads (*i.e.*, 500 μg to 3 mg) and essentially equivalent PC values at higher loads. However, neither the 250 mm × 10 mm I.D. columns nor the 250 mm × 22 mm I.D. column resolved completely the two major peaks of the TF1 peptide at loads greater than about 5 mg (data not shown).

Generally, columns with octadecyl ligands performed better than those with butyl ligands (*e.g.*, Fig. 3, symbols ○ and ■), and the performance of the columns packed with regular particles was better than that of the irregular particles (*e.g.*, Fig. 3, symbols △ and ◇). Although the peptides were relatively small (nineteen or twenty residues), the octadecyl 300 Å pore size silica columns performed better at lower loads than the correspondingly sized octadecyl 80 Å pore size silica column or the octyl 150 Å pore size silica column (Fig. 3, symbols ○, □, ● and ◇, respectively). At higher loads, column performance appeared to be independent of pore size.

Although chromatographic efficiency is directly proportional to column length,

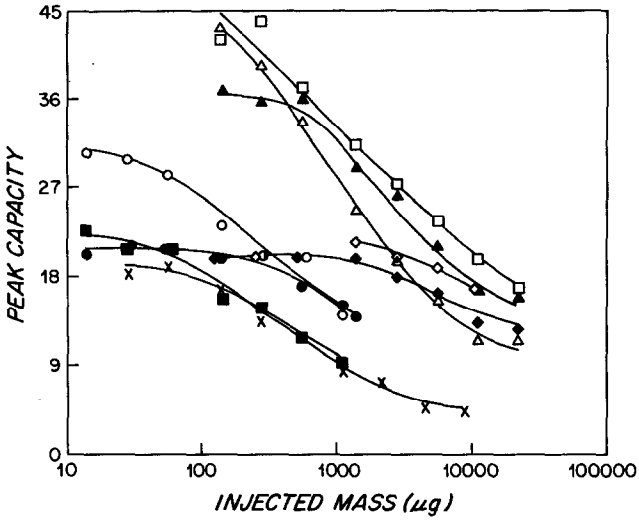

Fig. 3. Peak capacity *versus* injected mass for the evaluated columns. Details of the gradient conditions are given in Table II and the definition of peak capacity in Fig. 1. Solvent system: 0.1% trifluoroacetic acid aqueous phase, acetonitrile organic phase; gradient of 0–50% organic in 34 min with detection at 214 nm. The peptide used is TF1 (Table I). Specifications for each evaluated column: ● = 150 mm × 4.6 mm I.D. Vydac C_{18} HS, 5 μm particle diameter, 80 Å pore size; ○ = 150 mm × 4.6 mm I.D. Vydac C_{18} TP, 5 μm particle diameter, 300 Å pore size; ■ = 150 mm × 4.6 mm I.D. Vydac C_4 TP, 5 μm particle diameter, 300 Å pore size; □ = 250 mm × 10 mm I.D. Vydac C_{18} TP, 10 μm particle diameter, 300 Å pore size; ▲ = 250 mm × 10 mm I.D. Beckman C_3, 5 μm particle diameter, 300 Å pore size; △ = 250 mm × 10 mm I.D. Vydac C_4 TP, 10 μm particle diameter, 300 Å pore size; ◇ = 250 mm × 10 mm I.D. Rainin C_8, 12 μm particle diameter, 150 Å pore size; ◇ = 250 mm × 22 mm I.D. Vydac C_4 TP, 15–20 μm particle diameter, 300 Å pore size; × = 250 mm × 4.6 mm I.D. Vydac C_4 TP, 5 μm particle diameter, 300 Å pore size.

and although these data were obtained using equivalent optimal LSS gradients (as defined by LSS theory), the 250 mm × 4.6 mm I.D. C_4 TP column did not perform better than the 150 mm × 4.6 mm I.D. C_4 TP column (Fig. 3, symbols × and ■). Instead these columns showed essentially identical performance for the same injected mass.

DISCUSSION

The observed increase (~ 15%) in the recovery of peptides when 2-propanol was present in the organic phase is consistent with the observations of Wilson *et al.*[19], who found that an organic phase of 2-propanol gives from −5% to 30% higher recovery of proteins (MW 6500–69 000) than an acetonitrile mobile phase.

Further, 1% 2-propanol in acetonitrile has a significantly lower viscosity than neat 2-propanol, thus allowing a higher flow-rate for a given maximum pressure drop. This is particularly important in preparative appliations since mass throughput is related to flow-rate.

While Stadalius *et al.* [20] showed that, for a constant gradient volume, increasing the solvent velocity decreases the PC at low mass loads, we observed that at higher mass loads (> 100 μg) the PC was less dependent upon solvent velocity (data not

shown). In other words, during mass-overloaded operation, the decrease in PC attributable to increased solvent velocity is not significant compared to the decrease in PC due to the mass overload. Thus, by increasing the flow while maintaining a constant gradient volume, an increase in throughput can be achieved.

As shown in Fig. 2, increased injected mass caused retention decreases and some relative retention shifts in the gradient chromatography, which we presume to be due to isotherm curvature. Retention shifts with increased injected mass have been observed previously[5], are a major difficulty with the gradient RP-HPLC purification of crude synthetic peptides and have led to difficulties in relating preparative elution profiles to analytical profiles and in obtaining pure fractions.

Fig. 2 also demonstrates that superior mass loading performance was obtained with the TEAP aqueous phase, although this phase did not provide as much selectivity as the TFA phase and provided inadequate resolution of the two major peaks at injected masses greater than about 200 μg. Therefore, the use of a TFA phase appears to be superior for this separation since it resulted in better separation of these components. However, this conclusion is not valid for all peptides; for some peptides tested, the TEAP phase provided adequate resolution and hence would be preferred for its superior mass loading capability. Operationally, the TFA phase also afforded the advantages of fewer baseline irregularities peaks (Fig. 2g–i) and easier preparation.

As shown in Fig. 3, column overloading greatly reduced the higher efficiencies of small particle packings since the PC values of equivalent-length columns packed with 5- and 10-μm silicas were nearly the same. However, extension of this conclusion to larger particle sizes may not be correct since the column packed with irregular 12-μm particles (Table II, column 7) exhibited markedly poorer performance although this may reflect a difference in the efficiency of the packing. Therefore, in the gradient preparative mode, the 10-μm regular particles, which can be operated at a higher flow-rate for a given pressure drop than the 5-μm particles without sacrificing performance, are preferred.

Octadecylsilane-modified supports performed better than octyl-, butyl- or propylsilane-modified supports for equivalent column dimensions. Although in the overloaded state there was no apparent performance difference between the 300 Å and the smaller pore size supports the 300 Å pore size material is preferred since it offers superior performance at low loads[21].

The 250 mm × 10 mm I.D. columns showed a clear advantage for the purification of 1–10 mg of material, although based on previous work, the estimated capacity is expected to be nearly 100 mg[9]. Therefore, the column of choice for the gradient RP-HPLC purification of small amounts of peptides appears to be a 250 mm × 10 mm I.D. column packed with an octadecylsilane-modified 10-μm wide-pore support.

While the selection of this preferred column is based on the column sizes evaluated, the observation that the 250 mm × 4.6 mm I.D. and 150 mm × 4.6 mm I.D. columns showed the same mass loading behavior suggests that in gradient operation the mass load capacities of a column are primarily determined by the column radius, not the total amount of silica in the column. This, in turn, suggests that the use of short, wide columns for gradient preparative applications may be advantageous, as Pearson[22] has already demonstrated for the isolation of proteins. Alternately, DC and related techniques provide more effective utilization of the available stationary phase

in preparative applications than does conventional gradient RP-HPLC[10,11] and therefore may be preferable for this application.

Using the LSS optimal gradient volume and attempting to perform a preparative separation of a crude synthetic peptide without initial chromatographic optimization of conditions resulted in a operating mass capacity of 1–10 mg on a 250 mm × 10 mm I.D. column, which is 1–2 orders of magnitude *less* than is possible[9]. Fig. 3 clearly shows that not even the 250 mm × 22 mm I.D. column (when operated with the LSS optimal gradient volume) had sufficient capacity to separate a 20-mg crude synthetic peptide sample with PC > 20.

While this discouraging result would appear to support the conclusion that LSS theory does not provide an algorithm for the empirical prediction of appropriate preparative gradient conditions, examination of the meaning of "optimum" in LSS reveals an explanation for the greatly reduced mass loads. The LSS optimum gradient volume is chosen as representing the best compromise between the obtained peak capacity and the required time of analysis[12,13]. While this is entirely reasonable for an analytical separation, in a preparative application, it is desirable to maximize the mass throughput, which may require operation at less than maximum peak capacity or at longer separation times. This is consistent with the published results showing larger sample capacities than obtained here (*e.g.*, ref. 9); the gradient volumes used, if translated into gradients equivalent to those used herein, are significantly larger than the LSS optimum gradient volume, *i.e.*, a tradeoff of time for mass throughput.

In order to develop the sought-after algorithm, it is necessary to redefine "optimum" to include mass throughput and to extend the current LSS model to include the effects of mass-overloaded operation, including isotherm non-linearity, solution non-ideality, etc. While the consistency of our results with those obtained for proteins[18] indicates that this may be possible, the success of DC in efficiently utilizing the entire stationary phase suggests that an empirical model for predicting a DC-oriented purification procedure for crude synthetic peptides is more likely to allow sufficiently rapid purification of the products of multiple-peptide syntheses than is gradient RP-HPLC.

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