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PURIFICATION OF SYNTHETIC ANALOGUES OF YEAST MATING HORMONE BY REVERSED-PHASE CHROMATOGRAPHY

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SUMMARY

The α-type cells of *Saccharomyces cerevisiae* secrete low-molecular-weight peptides, termed α-factors, which affect the sexual conjugation between α- and a-mating types of this yeast. We have synthesized the tridecapeptide α-factor (Trp-His-Trp-Leu-Gln-Leu-Lys-Pro-Gly-Gln-Pro-Met-Tyr) and the dodecapeptide α-factor (His-Trp-Leu-Gln-Leu-Lys-Pro-Gly-Gln-Pro-Met-Tyr) and a series of eight analogues, without purification of intermediates, using standard solution phase techniques of peptide synthesis. Crude peptides (125–500 mg) were loaded on to a preparative μBondapak C_{18} column (Waters Prep LC/System 500) and eluted with methanol–water–trifluoroacetic acid mixtures. The recovery of purified peptide was as high as 93%. Mating factor analogues had biological activity similar to that of the natural peptides. The incorporation of trifluoroacetic acid (TFA) ($\leqslant 0.025\%$) in the mobile phase provides excellent conditions for the separation and purification of peptides. TFA has a significant effect on both peak shape and retention time in the concentration range 0–0.25%.

INTRODUCTION

The synthesis in reasonable amounts of highly pure biologically active peptides is of interest to many investigators. Purification procedures used in the past such as gel filtration and ion-exchange chromatography are generally slow, give poor resolution and may result in low yields of products. More recently, reversed-phase high-performance liquid chromatography (HPLC) has been applied effectively for the analytical and preparative separation of underivatized peptides. We report here a method for rapidly and efficiently purifying biologically active synthetic peptides of

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medium size (*ca.* 2000 daltons) in amounts in excess of 50 mg. The method involves the use of trifluoroacetic acid, which is volatile and easily removable, in a methanol–water mobile phase. Trifluoroacetic acid has been used previously as a modifier in the HPLC of peptides and proteins[1–4].

EXPERIMENTAL

A Waters Assoc. (Milford, MA, U.S.A.) HPLC system was used for all analytical separations. This consisted of an M-6000A solvent delivery unit and a U6K universal liquid chromatography injector, coupled to a Waters 450 variable-wavelength UV monitor with an 8-μl flow-through cell and coupled to an Omniscribe two-channel recorder (Houston Instruments, Austin, TX, U.S.A.). A μBondapak C_{18} column (10 μm, 30 × 0.39 cm I.D.) (Waters Assoc.) was also used. Samples were injected with a 25-μl Hamilton syringe. Solvents were of HPLC grade (Fisher Scientific, Pittsburgh, PA, U.S.A.) and water was glass distilled. Trifluoroacetic acid (Aldrich, Milwaukee, WI, U.S.A.) was of reagent grade and used without purification. Generally a flow-rate of 3 ml/min was maintained and separations were performed at room temperature. Sample sizes varied from 1 to 10 μg of peptide material injected in volumes of 1–20 μl. Detection was effected at 220 nm with a sensitivity of 0.1 a.u.f.s.

Preparative separations were carried out on a Waters Assoc. Prep LC/System 500 instrument. A Waters Assoc. Prep PAK 500-C_{18} cartridge (80 μm mean particle size, 30 × 5.7 cm) was used at a flow-rate of 200 ml/min. Solvents were of reagent grade and were filtered (Millipore, 0.45 μm) before use.

Peptides were synthesized by standard solution phase techniques using mixed anhydride and hydroxybenzotriazole-catalyzed active ester coupling methods. All unprotected peptides were isolated as the trifluoroacetate salts. Details of a similar synthetic strategy have been published previously[5] and full details on the synthesis of compounds characterized in this work will be published elsewhere.

Biological activities of the mating factors and analogues were determined by the "Shmoo" assay, which measured the formation of morphologically aberrant cells[5].

Amino acid analyses were performed at the Weizmann Institute of Science, Rehovot, Israel. Analyses were conducted using a Durrum amino acid analyzer and a one-column system. Peptides were hydrolyzed in sealed tubes using 6 *M* hydrochloric acid at 100°C for 24 h. For peptides containing methionine, anisole was added as a scavenger.

*Sample procedure: purification of synthetic Cha², Orn⁶-dodecapeptide α-factor**

Crude synthetic Cha²,Orn⁶-dodecapeptide α-factor (400 mg) was dissolved in 10 ml of the eluent (methanol–water–trifluoroacetic acid, 360:640:0.25) and injected onto a C_{18} column of the Prep LC/system 500, which had been equilibrated with the same solvent system. The column was eluted and the fractions were analyzed side by side using analytical HPLC. When all the impurities had been eluted, and a peak corresponding to α-factor was observed, the eluent was changed to methanol–water–

* Cha = β-Cyclohexylalanine.

trifluoroacetic acid (420:580:0.25) and further fractions were collected. The fractions containing pure α-factor were pooled and evaporated *in vacuo* at room temperature. The residue was dissolved in water (10 ml), filtered through a microfilter (0.45 μm) and the filtrate lyophilized. The yield was 153 mg and the purity as judged by analytical HPLC was 99.4%; $[\alpha]_D^{25} = -52.27$ (*c* 0.22, acetic acid). The final product was homogeneous on silica thin layers using 1-butanol–acetic acid–water (4:1:5, upper phase) and 1-butanol–acetic acid–water–pyridine (15:3:12:10) as the eluent.

RESULTS AND DISCUSSION

The α-type cells of *Saccharomyces cerevisiae* secrete a low-molecular-weight peptide termed the α-factor, which affects the sexual conjugation between the α- and a-mating types of this yeast. The sequence of two peptides which exhibit equivalent α-factor activity are (i) Trp-His-Trp-Leu-Gln-Leu-Lys-Pro-Gly-Gln-Pro-Met-Tyr (tridecapeptide α-factor) and (ii) His-Trp-Leu-Gln-Leu-Lys-Pro-Gly-Gln-Pro-Met-Tyr (dodecapeptide α-factor)[6]. In our laboratory, we have synthesized the tridecapeptide α-factor, the dodecapeptide α-factor and a series of eight analogues of the dodecapeptide by solution phase methods of peptide synthesis.

Couplings were accomplished either by the mixed anhydride or accelerated active ester technique. The major advantage of solution phase peptide synthesis compared with the solid phase technique is the certainty that the final peptide is void of errors or deletions in sequence. The major disadvantage is the time required for solution phase synthesis. Much of the time expended in preparing biologically active peptides, whether by solution or solid phase techniques, is consumed by the purification procedures. During our synthesis of the tridecapeptide mating factor and the series of dodecapeptide mating factors we omitted the purification of most intermediates. Despite this strategy we were able to purify the crude unprotected mating factors to >98% homogeneity in one simple and rapid operation (Fig. 1). The crude peptides (125–500 mg) were loaded onto a μBondapak C_{18} column (Waters Assoc. Prep PAK 500-C_{18}-cartridge, Waters Prep LC/System 500) and eluted with methanol–water–trifluoroacetic acid mixtures at a flow-rate of 200 ml/min. The fractions containing pure peptide were combined, concentrated to a small volume, filtered and then lyophilized. Fig. 2A shows an analytical elution profile of a crude octapeptide (Trp-His-Trp-Leu-Gln-Leu-Lys-Pro) that reflects optimal resolution in the methanol–water–trifluoroacetic acid system. This mobile phase was used to load and purify 470 mg of crude material. Fig. 2-B and C represent the analytical profiles of the purified products after lyophilization. Alternatively, a step-gradient may be employed to reduce the amount of solvent necessary for elution. Thus the time required to recover pure peptide will be significantly shortened (see Experimental). With Cha[2],(ε-biotinyl)Lys[6]-dodecapeptide α-factor (Fig. 3), methanol–water–trifluoroacetic acid (410:590:0.25) was used as the mobile phase to load the column and elute impurities. After impurities had been removed (as indicated by the analytical profiles of the fractions) the mobile phase was changed to methanol–water–trifluoroacetic acid (440:560:0.25). Using these methods, we purified both the tridecapeptide and dodecapeptide mating factors, eight analogues (Table I) of the dodecapeptide mating factor and the amine terminal octapeptide segment of the tridecapeptide α-factor. In addition to being homogeneous on reversed-phase HPLC using two mobile phases,

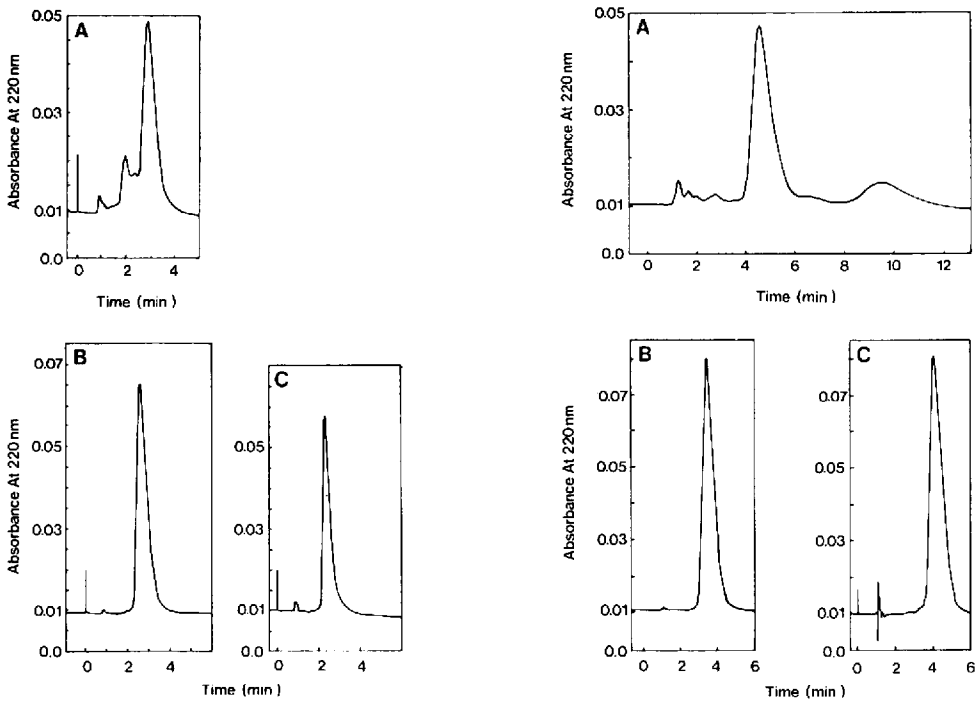

Fig. 1. Analytical elution profile of Cha^2,Orn^6-dodecapeptide α-factor using a μBondapak C_{18} support at a flow-rate of 3 ml/min. (A) Crude Cha^2, Orn^6 analogue before purification. Eluent: methanol–water–trifluoroacetic acid (420:580:0.25). (B) Purified Cha^2, Orn^6 analogue. Eluent: methanol–water–trifluoroacetic acid (420:580:0.25). (C) Purified Cha^2,Orn^6 analogue. Eluent: acetonitrile–water–trifluoroacetic acid (120:380:0.1).

Fig. 2. Analytical elution profile of crude amine terminal octapeptide fragment of the tridecapeptide α-factor using a μBondapak C_{18} column at a flow-rate of 3 ml/min. (A) Crude octapeptide before purification. Eluent: methanol–water–trifluoroacetic acid (380:620:0.25). (B) Purified octapeptide. Eluent: methanol–water–trifluoroacetic acid (390:610:0.25). (C) Purified octapeptide. Eluent: acetonitrile–water–trifluoroacetic acid (220:780:0.2).

all peptides gave one UV-positive ninhydrin-positive spot on silica thin layers using two different eluents. Amino acid analyses (Table II) of the purified product were all consistent with the expected peptide. All of the synthetic α-factors and analogs were examined as to relative abilities to cause aberrant morphology ("shmooing") of *S. cerveisiae* 2180-1A. All samples caused the formation of "shmoos" although some differences in specific activity (the minimum amount of peptide required for shmooing) were noted.

The amount of peptide contained in overlapping fractions was small, obviating the need for recycling. An analysis for the dodecapeptide analogues indicated a recovery of purified product as high as 93%. The recovery was calculated by determining the number of absorbance units of peptide applied to and recovered from the column.

Generally, a few milligrams of peptide can easily be purified on semi-preparative reversed-phase columns. Larger samples (>1.0 g) have also been purified on Waters Assoc. Prep PAK 500-C_{18} cartridges[7]. However, the purification of biologi-

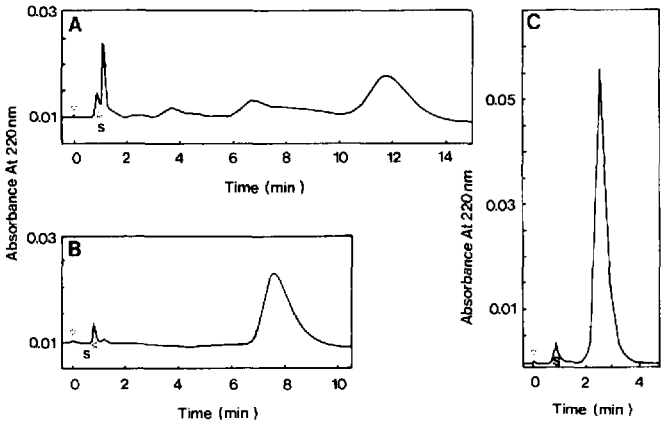

Fig. 3. Analytical elution profile of Cha^2,(ε-biotinyl)Lys^6-dodecapeptide α-factor using a μBondapak C_{18} column at a flow-rate of 3 ml/min. (A) Cha^2,(ε-biotinyl)Lys^6 analogue before purification. Eluent: methanol–water–trifluoroacetic acid (420:580:0.25). (B) Purified Cha^2,(ε-biotinyl)Lys^6 analogue. Eluent: acetonitrile–water–trifluoroacetic acid (120:380:0.1). (C) Purified Cha^2,(ε-biotinyl)Lys^6 analogue. Eluent: methanol water–trifluoroacetic acid (500:500:0.25).

cally active samples in the intermediate weight range is more problematic. Waters Assoc. do not recommend the use of the Prep PAK 500-C_{18} cartridge for samples of less than 1.0 g. Nevertheless, we have successfully purified more than fifteen different medium-sized peptides using the technique outlined in this paper.

In recent years the frequently encountered problems of peak broadening and long retention time during chromatography of peptides and proteins have been addressed with the use of various ion-pairing reagents. Hancock and co-workers used phosphoric acid[8] and ammonium or metal ion salts[9] for improving the resolution of peptides. Hirt *et al.*[10] and Voskamp *et al.*[11] described the use of trifluoroacetic acid in the analysis and purification of synthetic human calcitonin.

TABLE I

k' VALUES OF VARIOUS DODECAPEPTIDE MATING FACTOR ANALOGUES IN METHANOL–WATER–TRIFLUOROACETIC ACID

Column, μBondapak C_{18}; flow-rate, 3 ml/min; wavelength, 220 nm; 0.1 a.u.f.s.

Mating factor analogue	*Eluent proportions**	*k'*
Cha^2,Orn^6	42:58:0.025	2.3
Cha^2,Lys^6**	42:58:0.025	2.45
Cha^2,ε(acetyl)Lys^6	42:58:0.025	5.65
Cha^2,ε(biotinyl)Lys^6	42:58:0.025	10.6
Cha^2,Nle^6	42:58:0.025	17.4
Cha^2,Met^{11}**	38:62:0.025	5.9
Cha^2,D[α-^{2}H]Ala^8	38:62:0.025	7.4
Cha^2,Nle^{11}	38:62:0.025	10.8
N_{π}α-Dns-His^1,Cha^2	58:42:0.025	2.6

* Methanol–water–trifluoroacetic acid.

** Cha^2,Lys^6 and Cha^2,Met^{11} are the same compound but are presented in different notations so that a more direct comparison can be made.

TABLE II

AMINO ACID ANALYSIS OF α-FACTOR ANALOGUES

α-*Mating factor analogue*	*Molar ratio*											
	His	*Cha*	*Leu*	*Glu*	*Lys*	*Pro*	*Gly*	*Met*	*Tyr*	*Orn*	*Nle*	*D - / α - ^2H]Ala*
Cha^6,Orn^6	0.92	0.90	2.02	2.04	–	2.03	1.000	1.01	0.99	1.05	–	–
Cha^2,ε(acetyl)Lys^6-	0.95	1.03	2.06	2.06	1.03	2.05	1.000	1.02	1.01	–	–	–
Cha^2,ε(biotinyl)Lys^6-	1.04	0.92	2.08	2.06	1.06	2.18	1.000	1.01	1.02	–	–	–
Cha^2,Nle^6-	1.04	0.90	2.09	2.05	–	2.07	1.000	1.02	1.03	–	0.99	–
Cha^2,Nle^{11}	0.98	0.96	2.10	2.03	1.05	2.07	1.000	–	1.05	–	1.00	–
N-α-Dns-His^1,Cha^2-	–	1.19	2.13	2.19	1.18	2.21	1.000	0.99	1.01	–	–	–
Cha^2,D-[α-^{2}H]Ala^8-	0.94	0.92	2.14	2.06	1.09	2.03	–	0.97	0.98	–	–	1.00

In this investigation a series of dodecapeptides were efficiently purified using reversed-phase HPLC. Trifluoroacetic acid was an effective additive for improving the resolution and peak shapes during our peptide purification. The capacity factors (k') of various mating factor analogues (Table I) using methanol–water–trifluoroacetic acid mixtures are consistent with the overall hydrophobicities based on the component residues. Our choice of trifluoroacetic acid in preparative work was influenced by its low boiling point, which allows it to be easily removed from the peptide and eliminates a desalting step subsequent to HPLC purification.

The influence of trifluoroacetic acid on the mobility of peptides on μBondapak C_{18} columns depended on both the nature of the peptide and the trifluoroacetic acid concentration (Figs. 4 and 5). In the absence of trifluoroacetic acid, peptides containing free amino groups are strongly retained on reversed-phase columns using methanol–water as the mobile phase. The addition of small amounts of trifluoroacetic acid

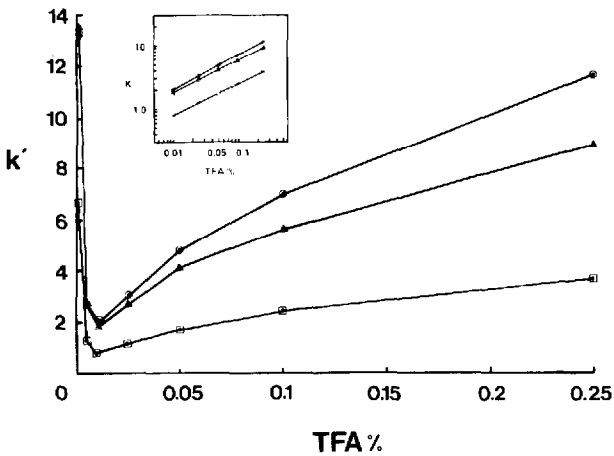

Fig. 4. Effect of trifluoroacetic acid (TFA) (%) on the k' values of mating factors (m.f.). □, Dodecapeptide m.f.; ○, Cha^2-dodecapeptide m.f.; △, amine terminal octapeptide. Insert: plot of log [trifluoroacetic acid, %] *vs.* log k'. Eluent: methanol–water (40:60). All runs were carried out on a μBondapak C_{18} column at a flow-rate of 3 ml/min.

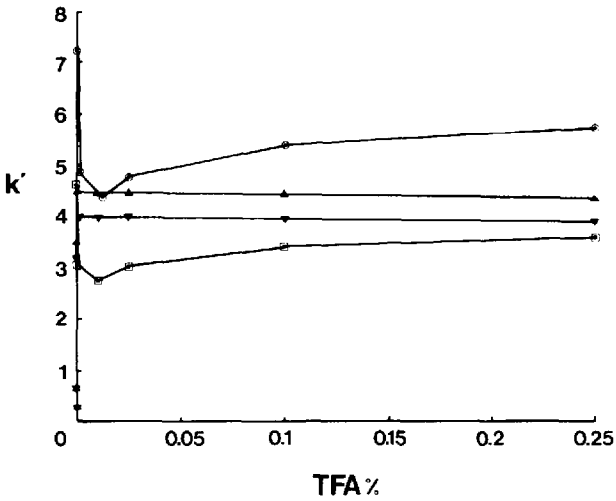

Fig. 5. Effect of trifluoroacetic acid (TFA) (%) on the k' values of partially protected peptides. □, TFA · Gly-Gln-Pro-Met-Tyr-OBzl; ○, TFA · Gly-Gln-Pro-Nle-Tyr-OBzl; ▽, Boc-Cha-Leu-Gln-Leu-Nle-Pro-OH; △, Boc-Cha-Leu-Gln-Leu-Orn(Z)-Pro-OH. Eluent for carboxyl terminal protected peptides, methanol–water (45:55); mobile phase for amine protected peptides, methanol–water (68:32). All runs were carried out on a μBondapak C_{18} column at a flow-rate of 3 ml/min.

to the mobile phase first causes a marked decrease in k' of the peptide. As additional trifluoroacetic acid is added to the mobile phase this effect reverses and at a specific trifluoroacetic acid concentration increased retention of the peptide is observed. The effect was observed even when the trifluoroacetic acid concentration was varied at constant pH (pH 3.0, data not shown). The specific trifluoroacetic acid concentration (0.01 %) above which additional acid results in higher k' values was the same for two dodecapeptide mating factors, the amine terminal octapeptide segment of the natural mating factor, and two pentapeptides with free amino termini. It is interesting that plots of log k' *versus* log [trifluoroacetic acid] are linear between 0.01 and 0.25% trifluoroacetic acid for the two dodecapeptides and the octapeptide which we investigated (Fig. 4, insert). The two peptapeptides with free amino termini also exhibit linear double logorithmic plots in this concentration range (data not shown). The dependence of the k' of the pentapeptides on trifluoroacetic acid concentration between 0.01 and 0.25 % is significantly less than that found for the larger peptides. This may reflect the fact that there is only one amino group on the pentapeptide whereas there are three amino groups on the octa- and dodecapeptides.

In contrast to the peptides with free amino groups, the mobility on a μBondapak C_{18} column of two N-protected hexapeptides was affected differently by addition of trifluoroacetic acid to the mobile phase. In the absence of trifluoroacetic acid the protected hexapeptides elute rapidly using methanol–water (68:32) as the eluent ($k' = 0.1$). Addition of small amounts of trifluoroacetic acid (up to 0.001 %) causes increased retention of the hexapeptides by the C_{18} reversed-phase column (Fig. 5). Increasing the trifluoroacetic acid concentration from 0.001 to 0.25% had no influence on the k' values of the N-protected hexapeptides (Fig. 5). We also examined the mobility of Boc-Leu-Orn(Z)-Pro-OH in methanol–water (38:62) and observed a

similar effect. For this N-protected tripeptide the k' changed from 1.7 in the absence of trifluoroacetic acid to $k' \gg 15$ upon addition of 0.001 % of trifluoroacetic acid.

In comparing the effect of fluoroacetic acid on peptides containing free ammonium groups to that on peptides with protected amino functions, it is clear that in the range 0.01–0.025 % of trifluoroacetic acid, the fluoroalkanoic acid has no influence on the k' of the latter compounds and markedly influences the k' of the former. Thus the carboxyl function of the peptide does not play a major role in influencing the HPLC profiles. The primary interactions in our system must involve the ammonium groups and the non-polar portions of the molecule.

The unprotected peptides contain three basic functional groups (two primary amines and the imidazole of histidine) all in their protonated forms as trifluoroacetate salts. Without trifluoroacetic acid in the mobile phase, these positively charged molecules interact strongly with the support and are not eluted from the column. The addition of small amounts of trifluoroacetic acid (up to 0.01 %) causes a reduction in the interaction between the peptide and the support, leading to the elution of the peptide and a decrease in the k' values. Although a conclusive explanation for the effect of small amounts of trifluoroacetic acid is not available, its addition may influence the interaction between ammonium groups on the peptide and residual siloxane or silanol[12] groups on the C_{18} support[13]. When the trifluoroacetic acid concentration is further increased (from 0.01 to 0.25 %) we observe an increased retention of the peptide characterized by increased k' values. This effect is consistent with an increased hydrophobic interaction between the peptide and the carbonaceous support, as would result from addition of a lipophilic ion pair reagent. Such ion-pairing effects have been reported previously for perfluoroalkanoic acids[2,3,14].

In the absence of added trifluoroacetic acid, N-protected peptides elute very rapidly in methanol–water mobile phases (Fig. 5). The increased retentions of the N-protected peptides at low trifluoroacetic acid concentrations are probably the result of ion suppression[9], an effect that shifts the ionic equilibrium and results in more undissociated carboxylic acid. The net effect is to increase the peptide solubility in the hydrophobic stationary phase, thereby leading to an increase in k'.

CONCLUSION

We have reported a method for rapidly and efficiently purifying (93 % recovery) medium-size peptides in excess of 50 mg using a Waters Assoc. Prep PAK 500-C_{18} cartridge with methanol–water–trifluoroacetic acid as the mobile phase. Trifluoroacetic acid has a significant effect on peak shape and retention time of amino deprotected peptides and shows the behavior expected for a hydrophobic ion-pairing reagent for concentrations of 0.01–0.25 %. It is ideally suited for preparative work because it is easily removed following purification. No adverse effect on equipment has been observed after 3 years of use and a μBondapak C_{18} column is still in excellent condition after 6 months of constant use.

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