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SEPARATION OF PEPTIDES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ON A WEAK ANION-EXCHANGE BONDED PHASE

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SUMMARY

Multicomponent peptide mixtures were separated by high-performance liquid chromatography on a MicroPak AX-10 column, a silica-based bonded-phase weak anion exchanger. A gradient of increasing 0.01 *M* triethylammonium acetate buffer (pH 6.0) into acetonitrile was usually used for elution. For peptides containing a number of acidic amino acids without compensating basic residues, such as delta sleep-inducing peptide, a dilute 0.04 *M* formic acid solution (pH 2.6) was employed as the eluent. Peptides of up to about 30 residues were successfully tested, including peptides such as somatostatin, neurotensin, ribonuclease s-peptide, α-endorphin, glucagon, and various angiotensins and bradykinins. Tryptic digests of horse heart cytochrome *c*, calmodulin and reduced and alkylated hen egg-white lysozyme were also successfully examined. Because of the volatility of the eluents used, peptides can be readily isolated for further investigation. Recoveries of over 80% were observed in those cases tested by comparative amino acid analysis.

INTRODUCTION

In the past several years, high-performance liquid chromatography (HPLC) has become one of the most important techniques for peptide separation. Reversed-phase HPLC, utilizing a variety of solvent systems[1–14], has been the type most widely used for this purpose. More recently, HPLC employing a macroreticular anion-exchange column was used to separate tryptic digestion mixtures of peptides[15].

We recently reported a method for the separation of dipeptides, including resolution of sequence isomeric and diastereoisomeric dipeptides, by HPLC on a weak anion-exchange bonded phase using mixtures of triethylammonium acetate (TEAA) buffer and acetonitrile for elution[16]. In this paper, we describe the separation

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of a large variety of peptides containing up to approximately 30 amino acid residues using a similar system. These peptides include somatostatin, α-endorphin, ribonuclease s-peptide, glucagon, various angiotensins and bradykinins as well as tryptic digestion mixtures of horse heart cytochrome *c*, calmodulin and reduced and alkylated hen egg-white lysozyme. This methodology has been developed for the study of the effects of ionizing radiation on peptides.

EXPERIMENTAL*

Apparatus

Separations were performed on a Hewlett-Packard Model 1084B liquid chromatograph (Hewlett-Packard, Avondale, PA, U.S.A.) equipped with a microprocessor, an automatic injector and a variable wavelength detector. The buffer solutions were filtered (Millipore, Bedford, MA, U.S.A.) prior to use. All separations were carried out on a 30 × 0.4 cm MicroPak AX-10 column (Varian, Walnut Creek, CA, U.S.A.), a difunctional weak anion-exchange bonded phase prepared on LiChrosorb Si-60 silica (10 μm)[17]. Prior to chromatography of most peptide mixtures, the column was first equilibrated with at least 50 ml of 0.01 *M* TEAA pH 6.0 buffer, to insure that column pH was also 6.0. Then, after column equilibration with the starting solution, usually acetonitrile–buffer (75:25), elution was begun with a gradient terminating at 100% buffer. Specific conditions are described in the Figures. For elution of acidic peptides, an isocratic flow of 0.04 *M* formic acid pH 2.6 buffer was employed. Again, the column was first pre-equilibrated with at least 20 ml of this eluent to insure that column pH was also 2.6.

Materials

Peptides were obtained from Sigma (St. Louis, MO, U.S.A.), Research Plus (Bayonne, NJ, U.S.A.) and Beckman (Palo Alto, CA, U.S.A.). Triethylamine was purchased from Eastman Kodak (Rochester, NY, U.S.A.) and purified by distillation. Buffer solutions were prepared by titrating 0.01 *M* acetic acid solutions with triethylamine to pH 6.0. Glass-distilled water and acetonitrile were purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Trypsin was obtained from Boehringer (Indianapolis, IN, U.S.A.).

Tryptic digestion

Tryptic digestions were carried out according to the procedure described by Margoliash[18]. 0.3 μmol of peptide were dissolved in 0.1 ml of 0.03 *M* Tris–HCl buffer (pH 8.0). 0.1 mg of trypsin was added and the mixture was incubated at 40°C for 24 h.

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* Certain commercial equipment, instruments, or materials are identified in this paper in order to adequately specify the experimental procedure. Such identification does not imply recommendation or endorsement by the National Bureau of Standards, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

Amino acid analysis

For recovery studies, the eluted peptides were dried *in vacuo* and hydrolyzed with constant boiling HCl in evacuated and sealed tubes at 110°C for 24 h. The hydrolyzates were analyzed on a Durrum D-500 amino acid analyzer.

RESULTS AND DISCUSSION*

The separation of a multicomponent peptide mixture is shown in Fig. 1, and peak identifications are given in Table I. Excellent peak symmetry for all peptides in this mixture was obtained with the exception of somatostatin (peak 1), which gave a tailing peak. Glucagon (peak 9) gave two peaks; amino acid analysis of collected material showed the same amino acid composition for both components. All of the other peptides in this chromatogram gave a single peak when freshly prepared solutions were individually injected. The 0.01 *M* TEAA pH 6.0 buffer used with acetonitrile in the above separations is the usual gradient system employed in peptide separation. It buffers well at the pH employed and is compatible with the pumps and column. It also allows sensitive detection of peptides at wavelengths in the range of 210–225 nm. Furthermore, this buffer is readily removed from eluted compounds by freeze-drying[19], facilitating the easy recovery of the separated peptides. Some pep-

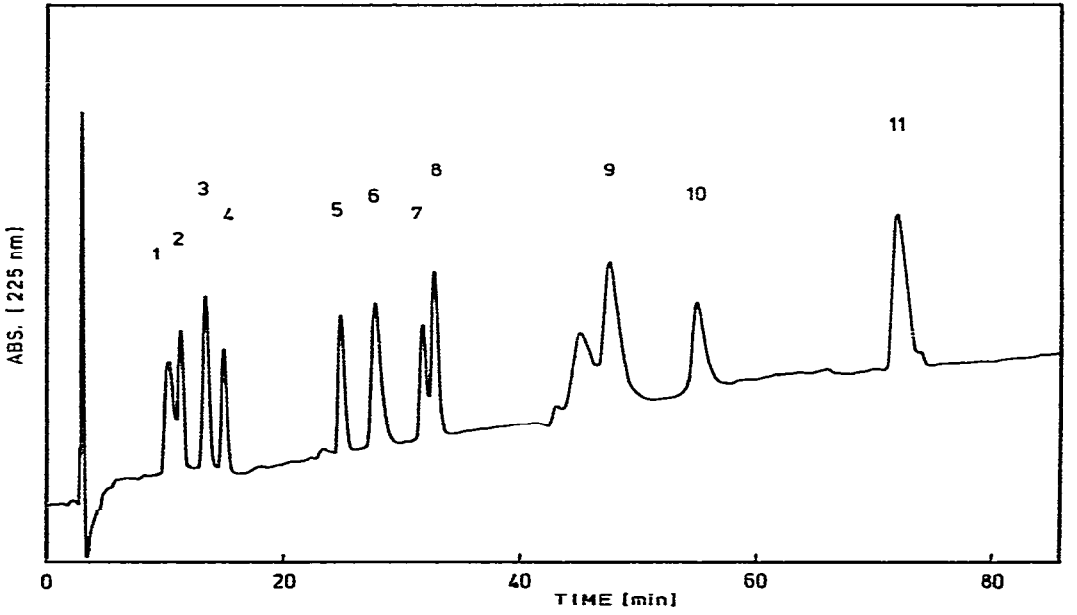


Fig. 1. Separation of various peptides. Column, MicroPak AX-10 (10 μm), 30 × 0.4 cm. Temperature, 30°C. Eluent: A, acetonitrile; B, 0.01 *M* triethylammonium acetate (pH 6.0), gradient program: linear starting from 25% B with a rate of 1% B per min. Flow-rate, 1 ml/min. Peak identification and sequences are given in Table I. Amount of injection, 0.5–5 μg per peptide.

* Abbreviations for amino acids follow IUPAC-IUB recommendations (see *Biochem. J.*, 126 (1972) 773).

TABLE I

PEAK IDENTIFICATION AND SEQUENCES IN FIGURES

Peak	*Peptide*	*Sequence*
Fig. 1		
1	Somatostatin	Ala–Gly–Cys–Lys–Asn–Phe–Phe–Trp–Lys–Thr–Phe–Thr–Ser–Cys
2	Proctolin	Arg–Tyr–Leu–Pro–Thr
3	Neurotensin	pGlu–Leu–Tyr–Glu–Asn–Lys–Pro–Arg–Arg–Pro–Tyr–Ile–Leu
4	Met–Enkephalin	Tyr–Gly–Gly–Phe–Met
5	Bradykinin potentiator C	pGlu–Gly–Leu–Pro–Pro–Gly–Pro–Pro–Ile–Pro–Pro
6	—	Lys–Glu–Thr–Tyr–Ser–Lys
7	α-Endorphin	Tyr–Gly–Gly–Phe–Met–Thr–Ser–Glu–Lys–Ser–Gln–Thr–Pro–Leu–Val–Thr
8	EAE-peptide	Phe–Ser–Trp–Gly–Ala–Glu–Gly–Gln–Arg
9	Glucagon	His–Ser–Gln–Gly–Thr–Phe–Thr–Ser–Asp–Tyr–Ser–Lys–Tyr–Leu–Asp–Ser–Arg–Arg–Ala–Gln–Asp–Phe–Val–Gln–Trp–Leu–Met–Asn–Thr
10	Ribonuclease s-peptide (RSP)	Lys–Glu–Thr–Ala–Ala–Ala–Lys–Phe–Glu–Arg–Gln–His–Met–Asp–Ser–Ser–Thr–Ser–Ala–Ala
11	IgE-peptide	Asp–Ser–Asp–Pro–Arg
Fig. 2		
1	Ribonuclease s-peptide	see above
2	IgE-peptide	see above
3	Glutathione oxidized form	γ-Glu-Cys–Gly | γ-Glu-Cys-Gly
4	—	Phe–Leu–Glu–Glu–Ile
5	DSIP	Trp–Ala–Gly–Gly–Asp–Ala–Ser–Gly–Glu
6	—	γ-Glu–Leu
7	—	γ-Glu–Glu
Fig. 3		
1	Angiotensin III (A III)	Arg–Val–Tyr–Ile–His–Pro–Phe
2	(Sar^1-Ala^8)-A II	Sar–Arg–Val–Tyr–Ile–His–Pro–Ala
3	A II	Asp–Arg–Val–Tyr–Ile–His–Pro–Phe
4	A I	Asp–Arg–Val–Tyr–Ile–His–Pro–Phe–His–Leu
5	(Val^5)-A II	Asp–Arg–Val–Tyr–Val–His–Pro–Phe
Fig. 4		
1	Bradykinin	Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg
2	Met, Lys–Bradykinin	Met–Lys–Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg
3	Lys–Bradykinin	Lys–Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg
Fig. 5		
1	—	Leu–Trp–Met–Arg–Phe–Ala
2	—	Leu–Trp–Met–Arg–Phe
3	—	Leu–Trp–Met–Arg
4	—	Met–Arg–Phe–Ala
5	—	Met–Arg–Phe
6	—	Arg–Phe–Ala
7	—	Leu–Trp–Met

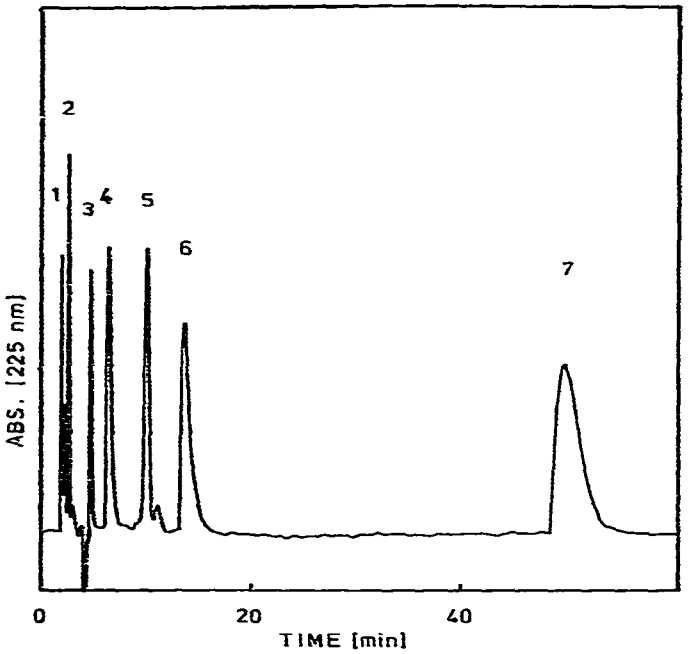
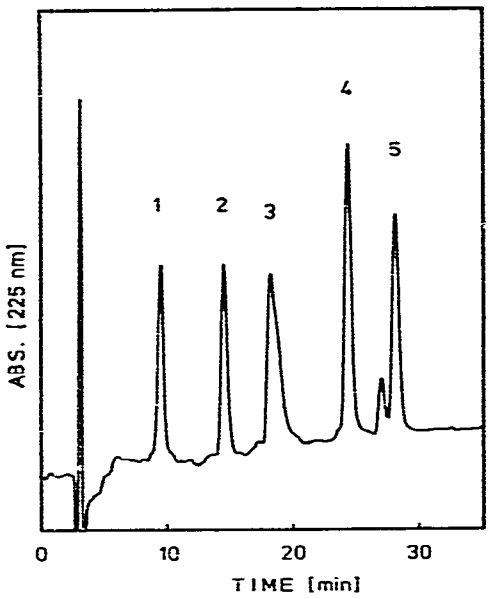


Fig. 2. Separation of some acidic peptides. Column as in Fig. 1. Temperature, 60°C. Eluent, 0.04 *M* formic acid (pH 2.6). Flow-rate, 1 ml/min. Peak identification and sequences are given in Table I. Amount of injection as in Fig. 1.

tides which contain a number of acidic amino acids with no compensating basic residues, such as delta sleep-inducing peptides (DSIP) (see Table I) or the dipeptide γ-Glu–Glu, have unacceptably long retention times when the elution conditions described in Fig. 1 are used. These peptides were chromatographed using an isocratic flow of dilute formic acid (pH 2.6) instead as the eluent, as is shown in Fig. 2. Both ribonuclease s-peptide (RSP) and IgE-peptide (peaks 1 and 2, respectively) could also be eluted with the solvent system described in Fig. 1. Although RSP contains three acidic amino acids (see Table I), it elutes much earlier than the other peptides in this mixture. This is probably due to the previously mentioned compensation of the acidic amino acids by the three basic amino acids or possibly steric hindrance to interaction of the carboxyl groups with the stationary phase. With increasing chain length, conformation of the peptide may also play a role. Thus, the pentapeptide with two Glu residues (peak 4) and DSIP (peak 5) elute later than RSP and IgE-peptide. As migth be expected from these other results, γ-Glu–Glu has the longest retention time. Dilute formic acid solutions are also compatible with the column and pumps used. In addition, formic acid is also volatile, allowing recovery of peptides for further use. As with the TEAA buffer, peptide recoveries are 80% or greater. The figures also demonstrate that sensitive detection of eluted peptides is also obtained with this eluent.

Separation of closely related peptides

The weak anion-exchange column is also capable of separating closely related peptides such as angiotensins or bradykinins. For example, Fig. 3 shows the separa-



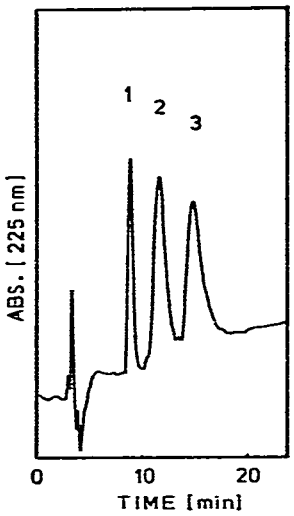


Fig. 3. Separation of some angiotensins. Column details as in Fig. 1. Peak identification and sequences are given in Table I. Amount of injection *ca.* 1 μg per peptide.

Fig. 4. Separation of several bradykinins. Column details as in Fig. 1 except gradient program: linear starting from 25% B with a rate of 1.7% B per min. Peak identification and sequences are given in Table I. Amount of injection, *ca.* 2 μg per peptide.

tion of a mixture of five angiotensins with excellent resolution, and all angiotensins gave symmetrical peaks. The peak representing angiotensin II showed a shoulder (peak 3) which corresponds to an impurity in this peptide. The small peak in front of (Val^5)–angiotensin II peak also represents an impurity.

The separation of three bradykinins is given in Fig. 4. Bradykinin gave a symmetrical peak whereas Met,Lys–bradykinin and Lys–bradykinin (peaks 2 and 3, respectively) yielded somewhat broad peaks.

Fig. 5 shows the separation of the hexapeptide Leu–Trp–Met–Arg–Phe–Ala (Research Plus Labs.) from the synthetic fragments that would be obtained by digestion with proteolytic enzymes or chemical cleavage. Excellent separation of these peptides was achieved by isocratic elution at 50°C.

Separation of tryptic digestion mixtures

Enzymatic digestion is frequently employed in sequence analysis of large peptides and proteins. For this reason, the weak anion-exchange column methodology was also employed to separate the peptides resulting from tryptic digestion of horse heart cytochrome *c*, calmodulin or reduced and alkylated egg-white lysozyme.

Fig. 6 shows the separation by gradient elution of the peptides resulting from tryptic digestion of horse heart cytochrome *c*. The elution became isocratic at 100% buffer after 60 min, and another four peaks were observed with continued flow of buffer. According to the sequence of horse heart cytochrome *c* given by Margoliash *et al.*[20], sixteen fragments and free lysine are expected upon digestion with trypsin.

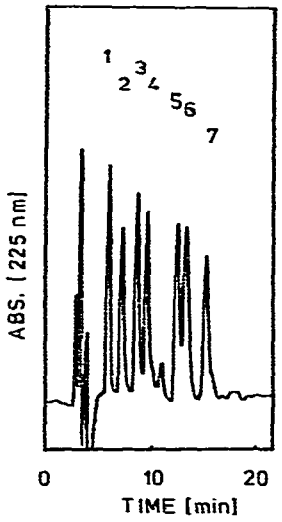


Fig. 5. Separation of Leu-Trp-Met-Arg-Phe-Ala and its simulated digestion fragments. Column and eluent as in Fig. 1. Isocratic elution with 21% B. Flow-rate, 1 ml/min. Temperature, 50°C. Peak identification and sequences are given in Table I. Amount of injection, *ca.* 1 μg per peptide.

Fourteen major peaks and some other small peaks were observed. Further work would be needed to characterize the separated fragments. The large peaks probably represent the fragments containing Tyr and Trp residues[20]. Since some digestion products contain a number of acidic amino acids, the separation of the above digestion mixture employing conditions described in Fig. 2 was also undertaken (Fig. 7). As expected, the majority of the fragments have no or little retention using these

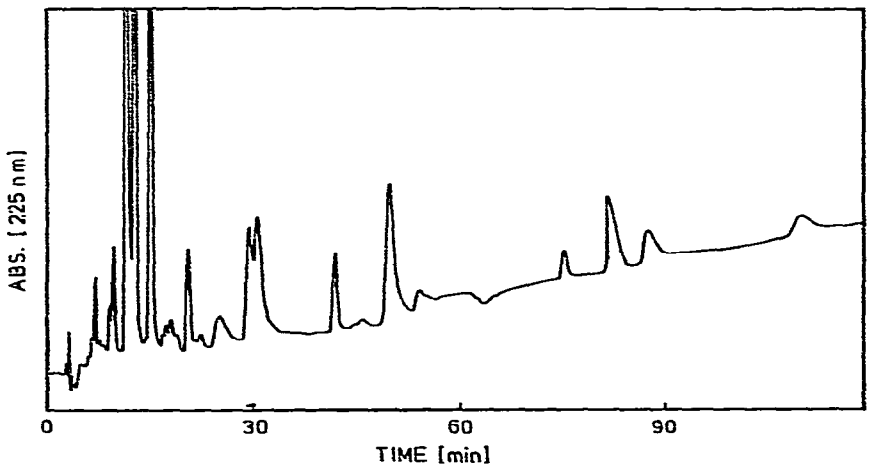


Fig. 6. Separation of a tryptic digest of horse heart cytochrome *c*. Column details as in Fig. 1 except gradient program: linear starting from 25% B with a rate of 0.6% B per min to 50% B then 4.5% B per min to 100% B. Amount of injection, *ca.* 10 nmol of cytochrome *c*.

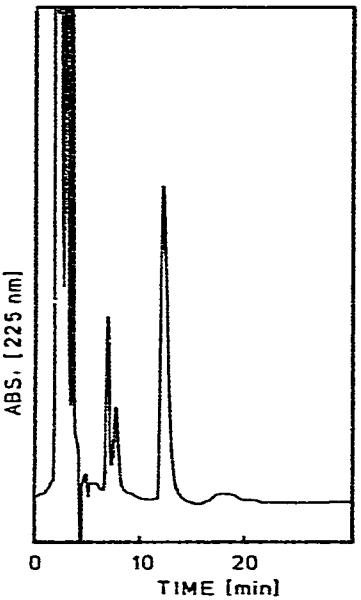


Fig. 7. Separation of a tryptic digest of horse heart cytochrome *c*. Column details as in Fig. 2. Amount of injection, *ca.* 10 nmol of cytochrome *c*.

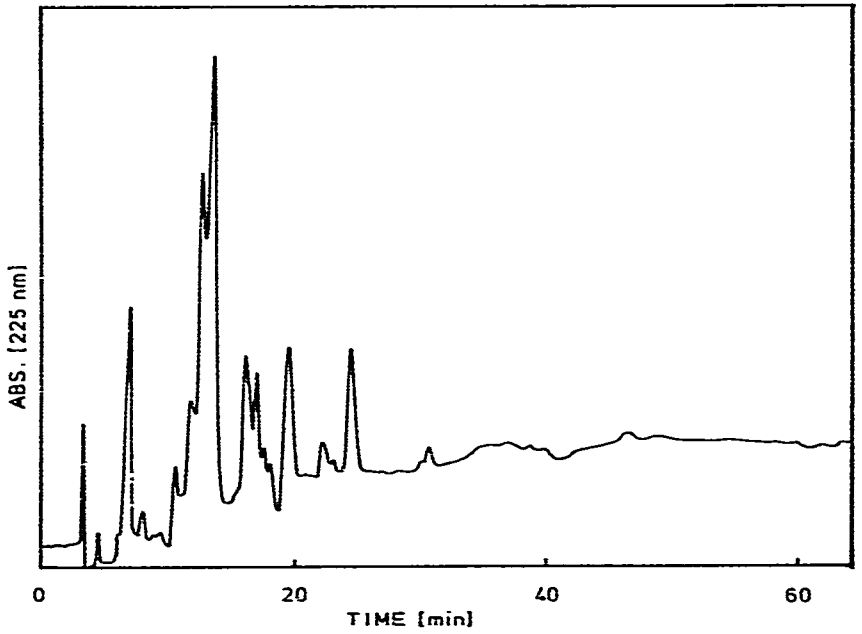


Fig. 8. Separation of a tryptic digest of calmodulin. Column details as in Fig. 1 except gradient program: linear starting from 25% B with a rate of 2% B per min. Amount of injection, *ca.* 15 nmol of calmodulin.

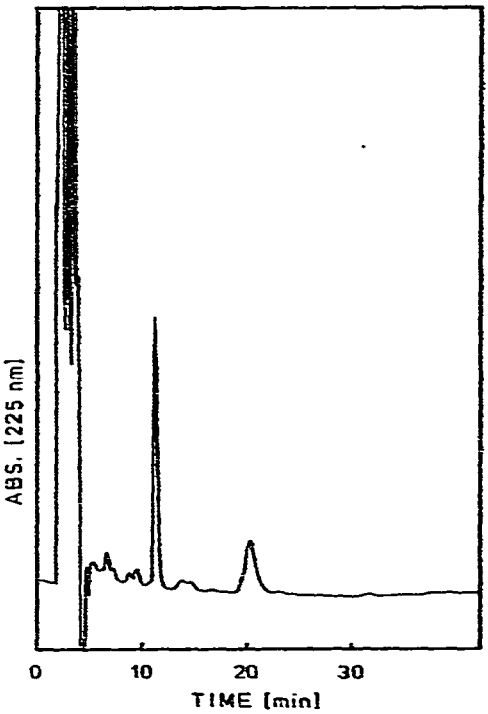


Fig. 9. Separation of a tryptic digest of calmodulin. Column details as in Fig. 2. Amount of injection as in Fig. 8.

conditions. However, in addition to these peaks, four other peaks were observed. The last peak in Fig. 7 may represent the fragment that contains four Glu residues and one Lys residue[20]. No other peaks were observed, even after an elution time of 90 min had elapsed. This data indicated that full separation of all peptides resulting from tryptic digestion of this protein was achieved.

The separation of the peptides from tryptic digestion of calmodulin is given in Fig. 8. Twelve fragments and free lysine are expected by digestion of calmodulin with trypsin[21]. Nine large peaks were observed besides material represented by small peaks. Fig. 9 shows the separation of this sample under the dilute formic acid conditions given in Fig. 2. In addition to the majority of the fragments with little retention, two more peaks were observed. Since tryptic fragments of calmodulin contain a large number of acidic amino acids, and the retention of such peptides shows a strong dependence on pH (see below *Effect of pH* and Fig. 11), a 0.07 *M* formic acid solution (pH 2.5) was also employed in an attempt to elute other possible fragments. However, no more peaks were observed after an elution time of 60 min.

Fig. 10 shows the separation of the tryptic digest of reduced and alkylated lysozyme. Again, the number of major peaks detected corresponds closely to the number of the fragments expected from tryptic digestion of lysozyme[22]. The two large peaks probably represent fragments with Trp and Tyr residues. Unlike the digests of cytochrome *c* and calmodulin, no other peaks in addition to those with no or litte retention, were observed when the dilute formic acid buffer was employed for elution.

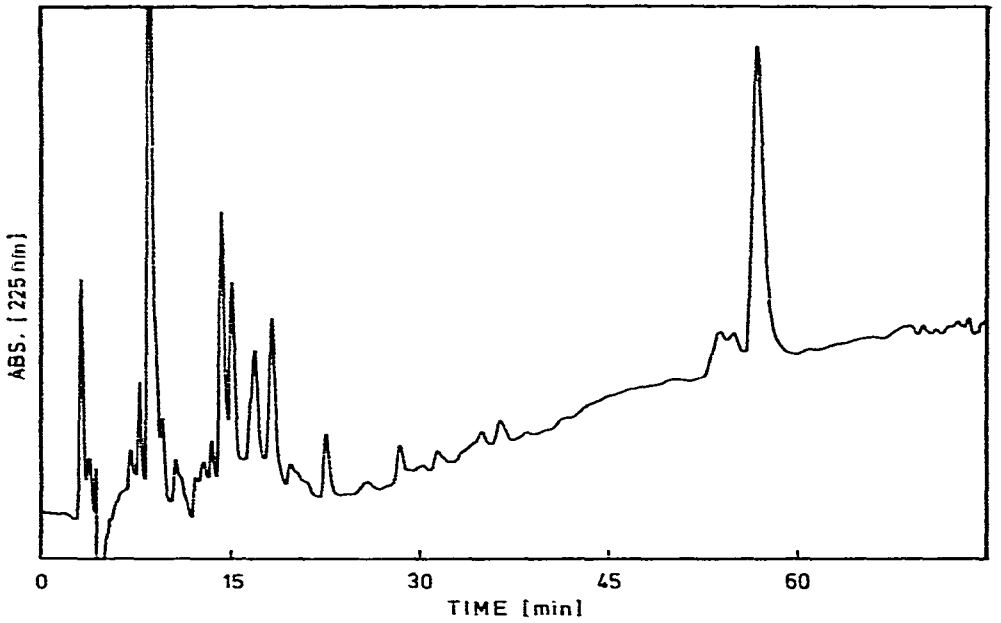


Fig. 10. Separation of a tryptic digest of reduced and alkylated lysozyme. Column details as in Fig. 1 except temperature: 40°C. Amount of injection, *ca.* 10 nmol of lysozyme.

Effect of temperature on retention and resolution

An increase in column temperature generally causes an increase in retention time. In some cases, resolution could be improved by increasing temperature to a certain limit starting from room temperature. For instance, the mixtures in Figs. 2 and 5 could be optimally separated at 60°C and 50°C, respectively. However, when gradient elution was used, an up-scale drift of the baseline was observed at temperatures above 40°C, due to higher UV absorption of the buffer. Nevertheless, baseline drift is still acceptable, and temperatures up to 60°C can be used to improve resolution of a given mixture if necessary.

Effect of pH

In a recent paper, we described separation of dipeptides using the TEAA buffer–acetonitrile solvent system, except the pH of the TEAA buffer was 4.3 (ref. 16). In the present work with peptides, a pH value of 6.0 was more suitable in terms of retention, peak symmetry and resolution. The separation selectivity of the stationary phase thus shows a quite strong dependence on the pH value of the eluent. This means that any pH value between 4 and 6 with this buffer system can be tried in order to improve resolution in a given separation using the chromatographic system described here.

The retention of acidic peptides even more strongly depends on the pH value of the eluent, as can be seen by the conditions described in Fig. 2 for their separation. This was further demonstrated in Fig. 11, where capacity factors (k') of DSIP were plotted *versus* pH of the eluent. In order to obtain a constant solvent strength, a 0.04 *M* formic acid solution was used and pH was changed by adding triethylamine to the

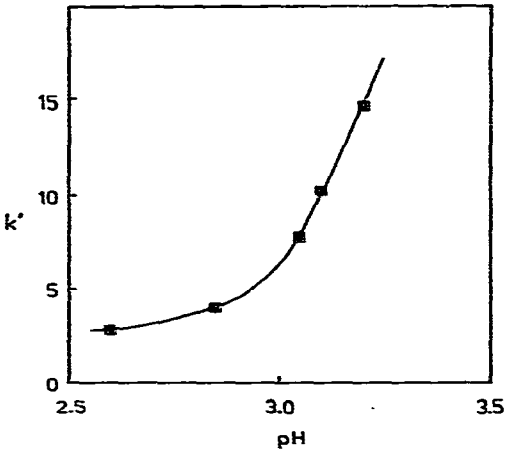


Fig. 11. Dependence of the k' value of DSIP (peptide No. 5 in Fig. 2) on the pH value of the eluent. Column as in Fig. 1. Eluent, 0.04 *M* formic acid, the pH value was adjusted by adding triethylamine. Flow-rate, 1.5 ml/min. Temperature, 60°C.

eluent. As can be seen from this plot, a very strong dependence of k' values on pH above ca. 2.9 was observed.

CONCLUSIONS

The results obtained in this paper clearly show that multicomponent mixtures of peptides or closely related peptides can be successfully resolved by HPLC on a weak anion-exchange bonded phase. The suggested method offers great advantages in terms of sensitivity, peak symmetry, reproducibility and high recoveries. The composition and pH of the mobile phase and the column temperature all contribute to resolution. The optimal conditions for resolution of a given separation problem can be determined by varying these parameters. Because the eluents used for elution are volatile, isolation of separated peptides for further use, such as structure determination, is facilitated. In addition, the experimental conditions appear to allow long column life (up to one year with several daily injections).

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