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ANALYTICAL AND PREPARATIVE MAPPING OF COMPLEX PEPTIDE MIXTURES BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Reversed-phase high-performance liquid chromatography (RP-HPLC) with a pyridine formate–1-propanol solvent system and fluorometric detection has been used to separate complex peptide mixtures. Excellent separations were obtained with mixtures containing a large number of relatively small peptides such as those generated by tryptic cleavage of proteins. RP-HPLC was also suitable for the separation of larger peptides such as those obtained, for example, by treating proteins with cyanogen bromide. The proposed method is rapid and simple, and it gives good peptide recovery. In addition, high detection sensitivity and large sample capacity make this technique suitable at both the analytical (1 nmol) and the preparative (1 μmol) level. The method is generally applicable to such problems as peptide mapping and protein sequencing.

INTRODUCTION

The separation of complex peptide mixtures generated by enzymatic cleavage of proteins has traditionally been carried out by such well established techniques as ion-exchange chromatography[1–3] and two-dimensional paper chromatography and electrophoresis[4]. Although these methods and their high-sensitivity adaptations[5–8] are still being successfully used for peptide mapping, or fingerprinting, and for protein sequence analysis, they are not without limitations. Often encountered problems are poor resolution and low recovery, particularly with larger peptides. Moreover, these procedures are frequently time-consuming and laborious.

In recent years reversed-phase high-performance liquid chromatography (RP-HPLC) has emerged as a new and powerful tool for the efficient separation of peptides (for a review see refs. 9 and 10) and even of small proteins[11,12]. The technique is simple, rapid and highly sensitive. Other characteristics are high capacity and ease of sample recovery. For these reasons, RP-HPLC is well suited for both analytical and preparative applications. Although the evidence that RP-HPLC is the most resolutive of the peptide separation methods is ample and its potential usefulness for the separation

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of peptides generated by enzymatic or chemical cleavage of proteins has already been shown by a number of investigators[13–23], detailed information on its practical applicability to a wide range of problems is not yet available. We have therefore undertaken to investigate whether RP-HPLC can be considered a generally useful method for analytical and preparative mapping of peptide mixtures and have examined such factors as resolution, recovery, capacity, sensitivity and size of peptides. In this report we present results which demonstrate that RP-HPLC is indeed a highly powerful and generally applicable method for the separation of complex peptide mixtures.

MATERIALS AND METHODS

Unless otherwise stated, all chemicals were reagent-grade (or best quality available) and obtained from major supply houses. In order to minimize residual amine content, formic acid and 1-propanol were distilled once, and pyridine twice, over ninhydrin (1 g/l). Acetonitrile (UV grade) was purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). The following proteins were used: bovine serum albumin (BSA); chicken ovalbumin; horse heart myoglobin; hen egg white lysozyme and porcine myelin basic protein (the latter protein a gift from Dr. F. Westall, Salk Institute).

An Altex Model 332 liquid chromatograph (Altex, Berkeley, CA, U.S.A.) equipped with an RP-18 column (25 × 0.4 cm, particle size 10 μm; Brownlee, Santa Clara, CA, U.S.A.) was used for most separation problems. The mobile phase used with this instrument was pyridine formate (0.36 *M* pyridine titrated to pH 3.0 with formic acid) containing propanol as described[13]. Samples were injected (in 0.5 ml 0.2 *M* acetic acid) by means of a 500-μl loop injector valve (Rheodyne, Berkeley, CA, U.S.A.) and were eluted at a flow-rate of 0.6 ml/min. The fluorescamine stream-sampling monitoring system, as previously described[24], was used for monitoring column eluates. A 5-μl aliquot of the column eluate was sampled every 10 sec by means of an automatic stream-sampling valve, transferred to the detection system with a water stream (14 ml/h) mixed with 0.4 *M* lithium borate pH 9.6 (28 ml/h) and fluorescamine (0.2 g/l, 14 ml/h; Pierce, Rockford, IL, U.S.A.) for detection in a fluoromonitor (American Instrument Co., Silver Spring, MD, U.S.A.). Under these conditions 5% of the total column eluate was used for detection.

For some separations a second HPLC system was used consisting of a Waters Model 204 liquid chromatograph equipped with two pumps, a M660 solvent programmer, a U6K sample injector (all from Waters Assoc., Milford, MA, U.S.A.), a variable wavelength UV detector (Schoeffel) and an RP-18 column (same type as above). The solvent system was 0.1 % (v/v) trifluoroacetic acid (TFA) in an acetonitrile gradient as described[25]. The flow-rate of the mobile phase was 0.7 ml/min. For injection, samples were dissolved in 0.5 ml 0.1 % TFA.

Performic acid oxidation[26], maleylation[27], cyanogen bromide cleavage[28] and trypsin digestion[29] of proteins were performed using standard methods as indicated. For amino acid analysis, a Liquimat III amino acid analyzer (Kontron, Zürich, Switzerland) equipped with a fluorescence detection system[30] was used.

RESULTS

Excellent separations of complex peptide mixtures were obtained by RP-HPLC using octadecylsilica (ODS, RP-18) columns and the pyridine formate–1-propanol solvent system. Typical separation patterns of tryptic digests of various proteins are shown in Figs. 1–4. Gradient elution with linear gradients of 2–4 h duration gave optimal separation of peptides having largely different hydrophobicities. With trypic digests of small proteins satisfactory resolution was obtained in runs of 2 h duration, while the more complex tryptic digests of large proteins required longer gradients (3–4 h) for adequate separations.

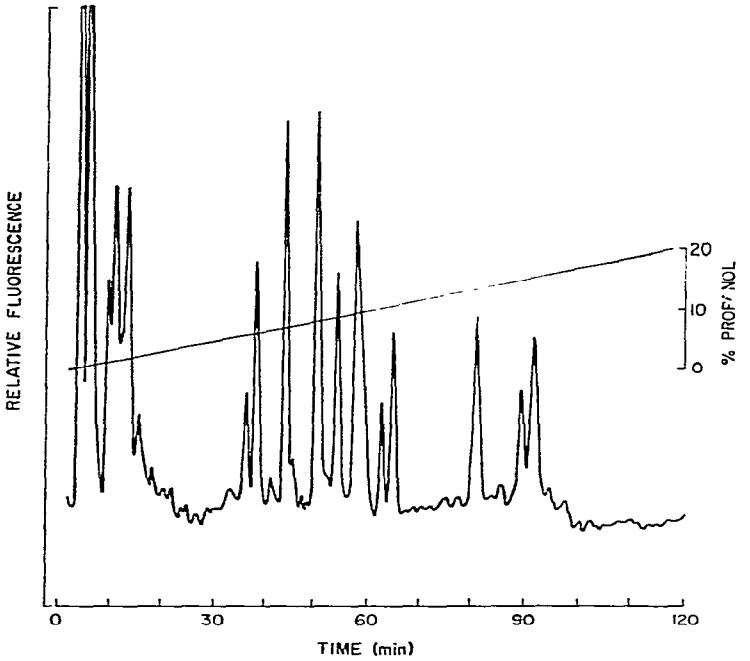

Fig. 1. RP-HPLC of a tryptic digest of 10 nmol (180 μg) performic acid-oxidized myelin basic protein using the pyridine formate–1-propanol mobile phase.

The high sensitivity of the fluorescamine stream-sampling detection system permits micropreparative chromatography with as little as 10 nmol of protein digest (Figs. 1,4) under conditions using no more than 5% of the column eluate for the detection reaction. High column capacity also permits the chromatography of relatively large amounts of protein digest. Fig. 5 shows an example of a separation of the tryptic peptides from 11 mg of lysozyme (corresponding to 750 nmol of each peptide). No loss in resolution was observed with this large quantity, as compared to a chromatogram obtained with a smaller amount (Fig. 2).

Peptide mixtures containing relatively large peptides (30–100 residues) can also be separated by RP-HPLC. Fig. 6 depicts the fractionation of peptides obtained by cyanogen bromide (CNBr) cleavage of myoglobin. Amino acid analyses (Table I) of

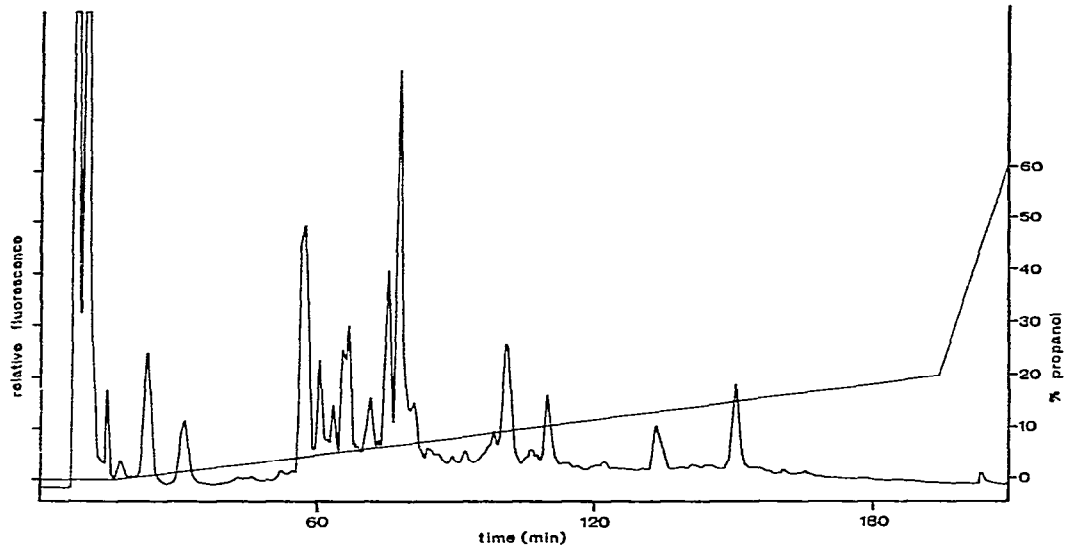

Fig. 2. RP-HPLC of a tryptic digest of 100 nmol (1.44 mg) performic acid-oxidized lysozyme using the pyridine formate–1-propanol mobile phase.

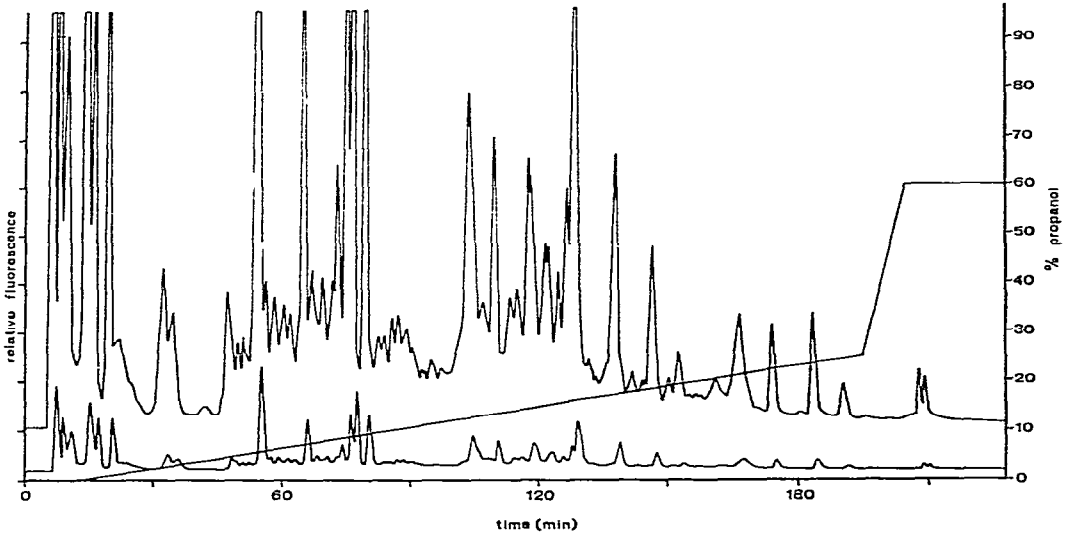

Fig. 3. RP-HPLC of a tryptic digest of 100 nmol (4.3 mg) performic acid-oxidized ovalbumin using the pyridine formate–1-propanol mobile phase. The lower trace is a 10-fold attenuation of the upper trace.

the major peaks confirmed that the expected fragments had been isolated. Furthermore, it revealed the presence of a large protein fragment which was not cleaved by CNBr. Another example of the separation of larger peptides is shown in Fig. 7. In this experiment performic acid-oxidized BSA was maleylated and then subjected to

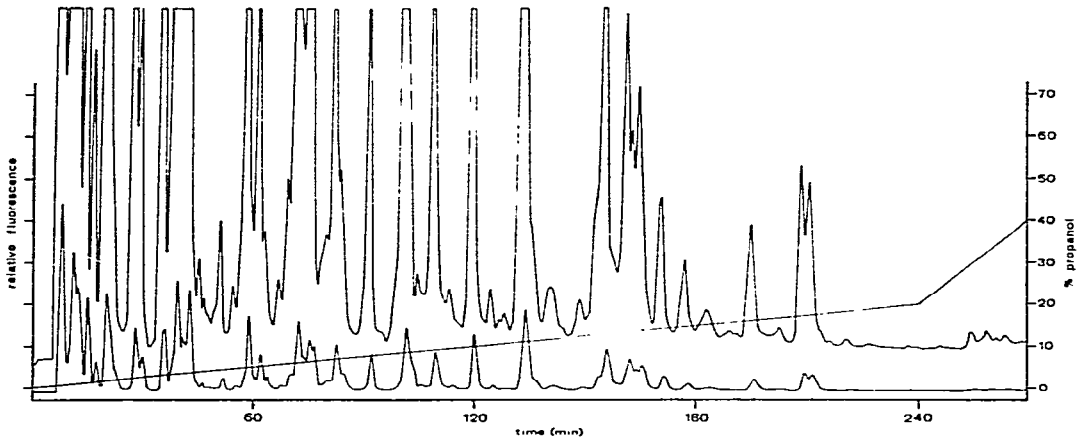

Fig. 4. RP-HPLC of a tryptic digest of 10 nmol (0.67 mg) performic acid-oxidized BSA using the pyridine formate–1-propanol mobile phase. The lower trace is a 10-fold attenuation of the upper trace.

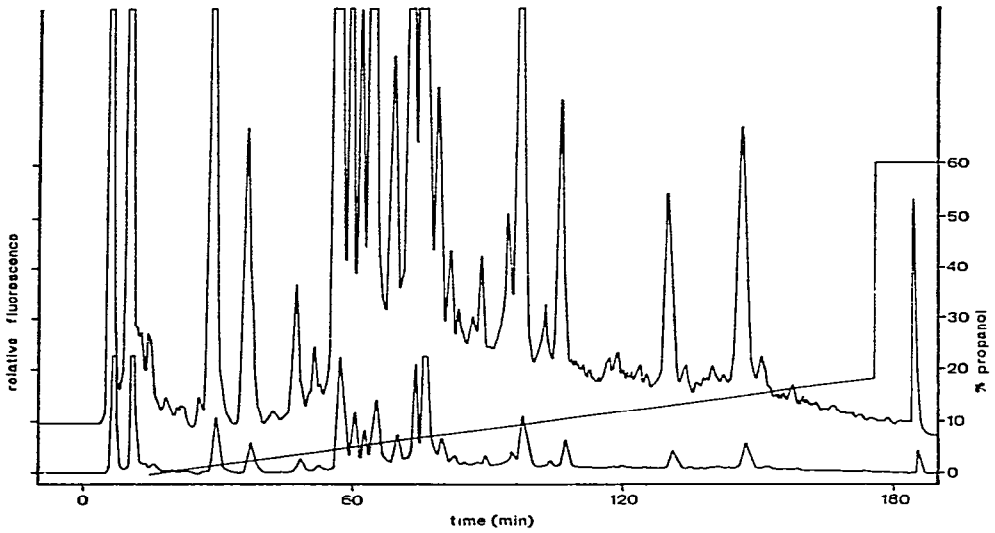

Fig. 5. RP-HPLC of a tryptic digest of 750 nmol (10.8 mg) performic acid-oxidized lysozyme using the pyridine formate–1-propanol mobile phase.

digestion with trypsin. This procedure is known to cleave the protein only at arginine residues because trypsin does not hydrolyze peptide bonds at maleylated lysine. BSA contains 58 lysine and 25 arginine residues and therefore specific cleavage at arginine residues will produce approximately 25 tryptic peptides many of which have molecular weights in the range of 3000–10,000. As can be seen in Fig. 7, satisfactory resolution is obtained with complex mixtures containing such large peptides.

From the preceding figures it is apparent that many peaks are incompletely resolved. This is particularly so when tryptic digests of large proteins are chromato-

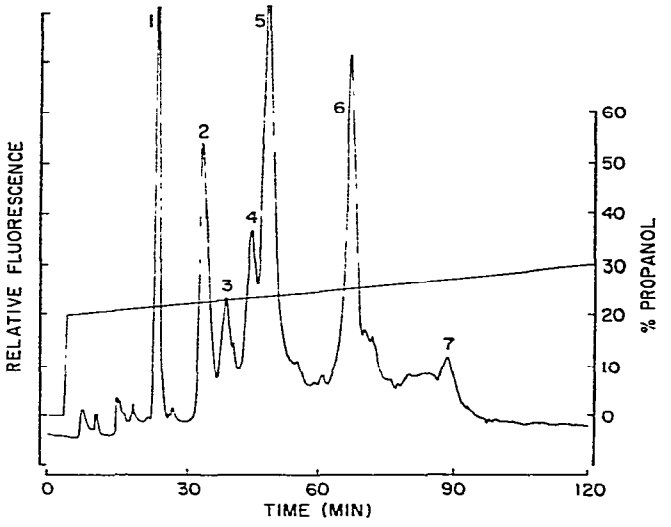

Fig. 6. RP-HPLC of the CNBr-peptides of 10 nmol myoglobin using the pyridine formate–1-propanol mobile phase.

TABLE I

AMINO ACID COMPOSITION OF CNBr-PEPTIDES OF MYOGLOBIN SEPARATED BY RP-HPLC (FIG. 6)

50–100 pmol aliquots of peptides were hydrolyzed in sealed, evacuated tubes in 6 *M* HCl containing 2% thioglycolic acid for 24 h at 110°C. Val, Ile and Leu values may be lower than expected due to incomplete hydrolysis under these conditions. Values are expressed as residues per molecule and are from single determinations. Values in parentheses are expected compositions of CNBr fragments. Peaks 1, 2, 5 and 6 correspond to myoglobin fragments 132–153, 56–131, 56–153 and 1–55, respectively.

Amino acid	*Peak 1*		*Peak 2*		*Peak 5*		*Peak 6*	
Asp	2.0	(2)	5.1	(4)	6.6	(6)	4.4	(5)
Thr*	1.1	(1)	3.3	(3)	4.2	(4)	3.4	(3)
Ser*	0	(0)	3.9	(4)	4.2	(4)	1.2	(1)
Glu	3.0	(3)	6.6	(6)	8.8	(9)	10.0	(10)
Pro**	—	(0)	—	(3)	—	(3)	—	(1)
Gly	2.1	(2)	7.0	(7)	8.6	(9)	5.6	(6)
Ala	2.7	(3)	8.8	(9)	11.5	(12)	3.4	(3)
Cys	0	(0)	0	(0)	0	(0)	0	(0)
Val	0	(0)	1.0	(3)	2.3	(3)	2.7	(4)
Met***	0	(0)	1.0	(1)	1.0	(1)	1.0	(1)
Ile	0.9	(1)	4.1	(6)	5.1	(7)	2.3	(2)
Leu	2.9	(3)	7.4	(8)	10.3	(11)	6.3	(6)
Tyr	1.0	(1)	0.6	(1)	1.7	(2)	0	(0)
Phe	2.0	(2)	2.2	(2)	3.5	(4)	3.0	(3)
His	0	(0)	7.2	(8)	6.8	(8)	2.9	(2)
Lys	3.3	(3)	9.5	(11)	14.1	(14)	4.4	(5)
Arg	1.1	(1)	0	(0)	1.2	(1)	0.9	(1)
Trp	0	(0)	0	(0)	0	(0)	2.0	(2)

* Uncorrected.
** Not determined.
*** Detected as homoserine and homoserine lactone.

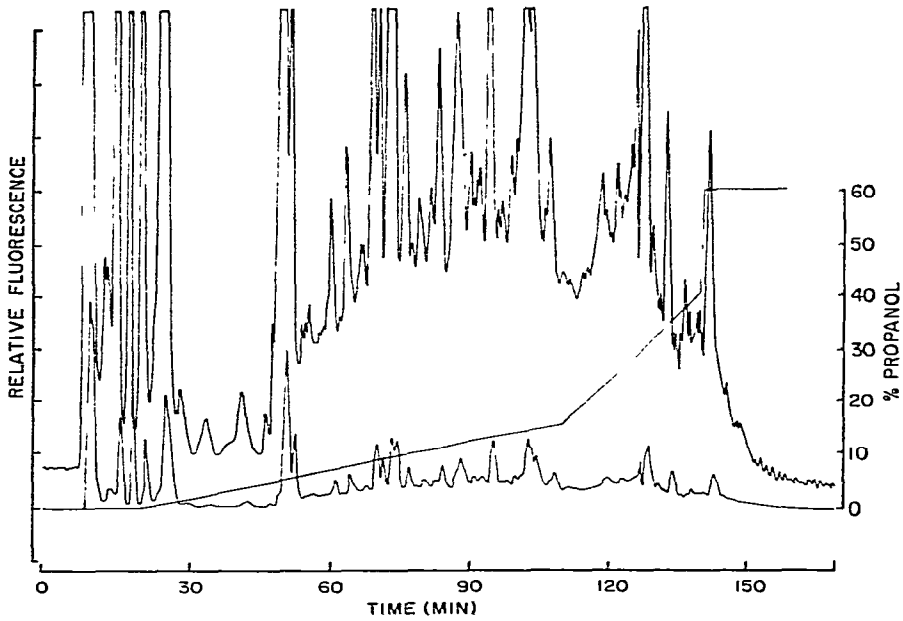

Fig. 7. RP-HPLC of a tryptic digest of 100 nmol (6.7 mg) performic acid-oxidized, maleylated BSA using the pyridine formate–1-propanol mobile phase. The lower trace is a 10-fold attenuation of the upper trace.

graphed. Due to the complexity of peptide mixtures generated by enzymatic cleavage of proteins, complete resolution of all peptides is not always possible under gradient conditions primarily chosen for the elution of all small and large, relatively hydrophobic as well as hydrophilic peptides. Often it is possible, however, to obtain satisfactory resolution by re-chromatography of partially resolved peaks under isocratic conditions, *i.e.*, at a constant concentration of the organic solvent in the mobile phase. Fig. 8 shows separations obtained by re-chromatography of incompletely separated peaks under various isocratic conditions. The best resolution is obtained with solvents having a relatively low content of organic modifier (Fig. 8C). Another approach to the separation of partially resolved peaks is exemplified in Fig. 9. While isocratic re-chromatography of unresolved peptides in the same solvent system was not very effective (Fig. 9B), the use of an alternative chromatography system (such as 0.1% TFA in a gradient of acetonitrile) yielded completely separated peaks (Fig. 9C) and revealed the presence of an additional peptide not apparent previously.

DISCUSSION

RP-HPLC appears well suited for the separation of complex peptide mixtures. It is clear, however, that complete resolution of all peptides is difficult to achieve in a single run, particularly when the mixture contains a large number of peptides as do tryptic digests of larger proteins. Gradients designed for the elution of a wide variety of largely different peptides are not suitable for the separation of peptides with similar chromatographic behavior. Although microprocessor technology now permits the

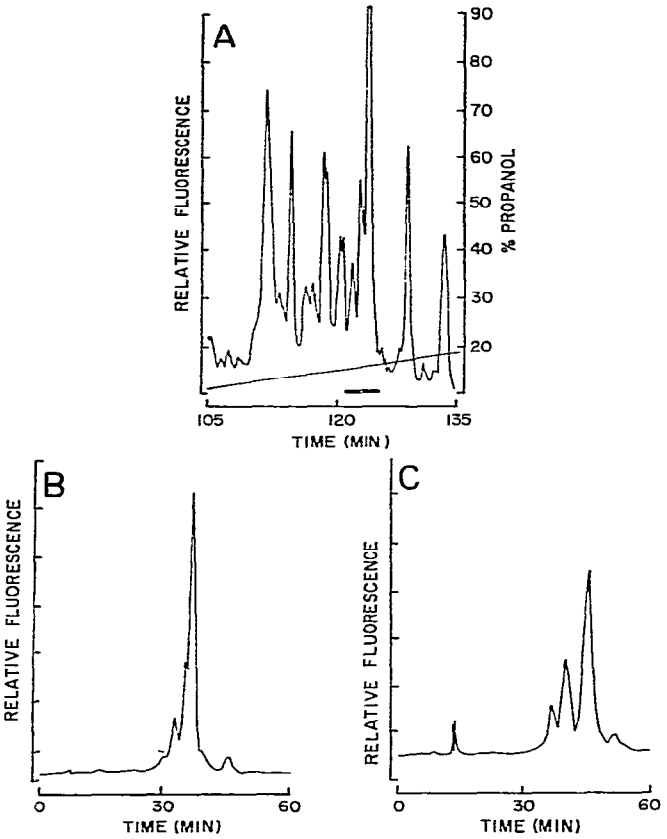

Fig. 8. Separation of partially resolved peptides by re-chromatography under isocratic conditions: A, RP-HPLC of a lysozyme digest (excerpt from Fig. 2); B, re-chromatography of a fraction (marked by a horizontal bar) in the same chromatographic system under isocratic conditions with 13% 1-propanol; C, same as in B with 10.5% 1-propanol.

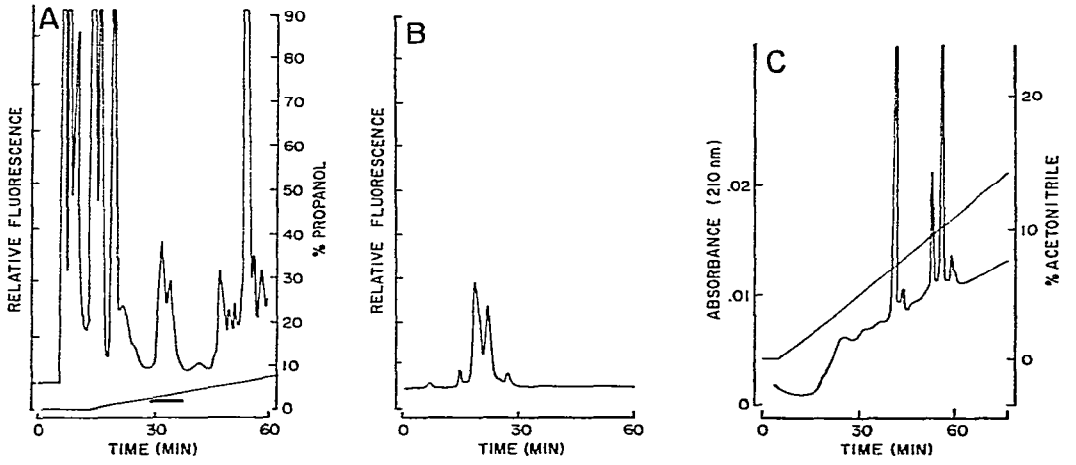

Fig. 9. Separation of partially resolved peptides using a different chromatographic system: A, RP-HPLC of a tryptic lysozyme digest in the pyridine formate–1-propanol solvent system (excerpt from Fig. 2); B, re-chromatography of a fraction (marked by horizontal bar) in the same system using isocratic conditions with 0% propanol; C, re-chromatography of the same fraction in the TFA–acetonitrile mobile phase.

modification of gradients for better separation of partially resolved peptides (*e.g.*, with multilinear or mixed isocratic/linear gradients), the design of a gradient optimally separating all peptides would be difficult and time-consuming in practice. It is better to use a general linear gradient and collect partially resolved peptides for re-chromatography. In some instances re-chromatography of the central zone of a peak under identical conditions may be sufficient to isolate a peptide from an incompletely separated neighboring peptide. A more useful approach is to re-chromatograph a zone of partially resolved peptides under isocratic conditions with the propanol concentration so adjusted that optimal separation is achieved. It is possible, however, that even isocratic elution will not resolve certain peptides. In this case, it has been our experience that an alternative mode of RP-HPLC with different solute selectivity is best suited. We have found the TFA–acetonitrile mobile phase to be very useful for the separation of peptides otherwise difficult to resolve. In contrast to the formate anion of the pyridine formate buffer, the trifluoroacetate anion forms relatively hydrophobic ion-pairs with positively charged side-chain functions of peptides[24]. Upon ion-pairing, the hydrophobicity of a peptide increases as a function of the number of positive charges. As with cation-exchange chromatography, the positive charges of peptides will consequently contribute to increased retention in hydrophobic ion-pairing RP-HPLC.

When tryptic protein digests are chromatographed in the pyridine formate–1-propanol system a large peak almost always appears at the beginning of the chromatogram. This peak contains amino acids and small polar peptides which are not retained when the column is eluted with pyridine formate buffer. The relatively high pyridine concentration (0.36 *M*) of the mobile phase may be responsible for the rapid elution of some polar small peptides in the void volume. Better resolution of such peptides may be obtained with mobile phases containing less pyridine (*e.g.*, 0.1 *M* pyridine titrated to pH 3.0 with formic acid) or with an alternative mobile phase such as TFA–acetonitrile.

The results of this study also indicate that large peptides, such as CNBr peptides or those present in the highly complex trypsin digest of maleylated BSA, can be efficiently separated by RP-HPLC using the pyridine formate–1-propanol system. The successful use of RP-HPLC in conjunction with this mobile phase for the separation of closely related large peptides has already been demonstrated by the separation of normal and mutant globin chains with single amino acid differences[12] and it may therefore be concluded that efficient separations of large peptides should generally be possible. In earlier work on RP-HPLC of large peptides (or small proteins) low recovery was cited as a potential problem[11,23,31]. We have determined the recoveries with a number of peptides and small proteins (molecular weight $<20{,}000$) and found them to be better than 90% in all cases (data not shown).

RP-HPLC is well suited for both analytical and preparative applications. It is especially useful in analytical (and micro-preparative) peptide mapping when only limited amounts of protein are available. As little as 1 nmol of peptide can be reliably detected with the fluorescamine stream-sampling detector with only 5% of the column eluate being used for detection. Sensitivity reaching well into the picomole range can be obtained by increasing the portion of column eluate used for detection. It is important to recognize the value of micropreparative peptide mapping in the low nanomole range because with modern techniques only picomole amounts of peptides are required

for amino acid analysis[30] and a few nanomoles may be sufficient for sequencing[32]. The power of the method has recently been demonstrated in our laboratory when micro-preparative peptide mapping was used for structural characterization of somatostatin-28, a novel hypothalamic peptide with somatostatin-like immuno- and bio-activity, of which only 4 nmol was available for mapping[32]. The proposed method can also be applied conveniently to preparative peptide mapping for the purpose of isolating peptides for sequence analysis with conventional methods. We have chromatographed as much as 11 mg of a protein digest (*i.e.*, 750 nmol of lysozyme, Fig. 5) on an analytical reversed-phase column and even larger quantities may be separated on a semi-preparative column.

The use of pyridine formate–1-propanol as the mobile phase has been found entirely satisfactory for peptide mapping by RP-HPLC. We have chosen this system because it has already been shown to be highly useful in a number of practical applications involving the isolation of peptides and proteins[13,20,21,32–37]. The system gives high recovery with large peptides; it can be coupled to the sensitive fluorescamine stream-sampling detection system and is volatile and therefore compatible with all preparative applications. One drawback of the detection system is that certain peptides cannot be detected with fluorescamine (those lacking a primary amino group; *e.g.*, peptides with proline or pyroglutamic acid at the N-terminal). This is not considered to be a serious problem because peptide mixtures generated by the usual enzymatic or chemical cleavage procedures rarely contain such peptides.

In conclusion, RP-HPLC compares favorably with existing techniques such as ion-exchange chromatography or two-dimensional chromatography–electrophoresis on paper or thin layers. RP-HPLC is considerably faster than these techniques. In contrast to the two-dimensional mapping techniques, it is much simpler and has higher capacity. Problems of peptide recovery which are common with conventional methods are virtually non-existent with RP-HPLC. These advantages should bring the method to play a prominent role in peptide mapping and isolation in the future.

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