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SEPARATION OF BASIC, HYDROPHILIC PEPTIDES BY REVERSED-PHASE ION-PAIR CHROMATOGRAPHY

I. ANALYTICAL APPLICATIONS WITH PARTICULAR REFERENCE TO A CLASS OF SERINE PEPTIDE SUBSTRATES OF CYCLIC AMP-STIMULATED PROTEIN KINASE

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SUMMARY

Basic, hydrophilic peptides exemplified by Arg-Arg-Ala-Ser-Val, a substrate of cyclic AMP-stimulated protein kinase, could be efficiently retarded by ion-pair formation with *n*-hexanesulphonic acid on a C_{18} column in phosphate buffer systems with ethanol as organic modifier. Reversed-phase ion-pair chromatography appears to be an ideal method for separating peptides of this type. Highly efficient separations under isocratic conditions are reported for peptides composed of 5–9 amino acids, with up to three basic residues, most having sequences derived from the phosphorylatable site of pyruvate kinase.

INTRODUCTION

This paper deals with the problem of separating basic, hydrophilic peptides by high-performance liquid chromatography (HPLC). Among such peptides we were especially interested in serine-containing ones, because they have previously been studied by us and others as substrates of cyclic AMP-stimulated protein kinase[1–5]. The corresponding cyclic GMP-stimulated enzyme has a similar substrate specificity[6,7]. A future paper in this series will deal with the phosphoserine peptides formed in these reactions.

Separation and analysis of peptides is often a difficult and time-consuming step. In the case of arginine- and lysine-containing peptides, the presence of basic groups can generally be exploited for purification purposes by electrophoresis and ion-exchange chromatography. Another interesting approach, pioneered by Schill and co-workers[8], is to generate ion pairs of peptides with hydrophobic anions and

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separate these complexes on straight or reversed-phase columns. Ion-pair HPLC has recently been reviewed[9]. This principle has recently been applied to analysis of the basic peptide somatostatin[10]. To our knowledge, however, it has not yet been used for basic, hydrophilic peptides. The major difficulty with this class of peptides is to retain them from the void volume. It is demonstrated in this paper that, by applying reversed-phase ion-pair HPLC, regulation of retention can be achieved within wide limits.

MATERIALS AND METHODS

Isocratic mobile phases were used consisting of binary aqueous solvents with ethanol as organic modifier. The buffers were prepared from orthophosphoric acid and sodium dihydrogenorthophosphate to an ionic strength of 0.1 *M*. Hexanesulphonate was used as counter ion in the mobile phase. 1-Hexanesulphonic acid was obtained from Eastman-Kodak Co. (Rochester, NY, U.S.A.). All substances and solvents were of analytical or reagent grade.

The peptides used as model substances in this work are summarized in Table I. They were all prepared in this laboratory by the solid-phase method of Merrifield[11,12] as briefly described[2]. Most of them were purified by ion-exchange chromatography on carboxymethyl cellulose[2] and their composition carefully checked by amino acid analysis after acid hydrolysis. Their high purity is confirmed in the present work.

The liquid chromatographic system consisted of a Waters Model 6000A solvent delivery device, a Waters U6K injector, and a Waters variable-wavelength detector (Waters Assoc., Milford, MA, U.S.A.). The detection wavelength was 210 nm at high sensitivity, which made possible the detection of peptide quantities at least as small as 0.1 nmol. In a typical experiment 1–2 nmol of each peptide was injected sequentially, dissolved in up to 5 μl of water.

The separation columns, 250 × 4 mm, were packed by the balanced-density slurry technique[13], with Spherisorb C_{18} (10 μm) as chromatographic support for reversed-phase HPLC. These columns were always preceded by a short pre-column. The Spherisorb support was obtained from Phase Separations (Queensferry, Clwyd, Great Britain). The support used in the pre-columns was Bondapak C_{18}/Corasil (Waters).

The separations were run at a nominal flow-rate of 1 ml/min, corresponding to 1.3 mm/sec, at room temperature (22°C) and the pH measurements performed at the same temperature.

RESULTS AND DISCUSSION

The structures of all peptides studied in this paper are given in Table I.

Fig. 1 shows the separation of M 67, M 66 and M 158 which from a structural point of view are reasonably similar and possess one, two and three arginine residues, respectively. In the presence of *n*-hexanesulphonic acid, the hydrophobicity of the ion-pair-containing complexes increases with the number of basic groups in agreement with prediction. The additional leucine residue in M 158 no doubt contributes to an increased hydrophobicity. The differences between the corresponding retention times are, however, considerable and give an indication of the power of this

TABLE I

PEPTIDE STRUCTURES

The peptides are numbered according to a local code. Peptides M 57–M 73 are numbered as in ref. 2. Gva = δ-Guanidinovaleric acid.

M 57	Leu-Arg-Arg-Ala-Ser-Val-Ala
M 66	Arg-Arg-Ala-Ser-Val-Ala
M 67	Arg-Ala-Ser-Val-Ala
M 69	Arg-Arg-Ala-Ser-Val
M 72	Leu-Arg-Ala-Ser-Val
M 73	Arg-Leu-Ala-Ser-Val
M 87	Val-Leu-Arg-Arg-Ala-Ser-Val-Ala
M 97	Gly-Val-Leu-Arg-Arg-Ala-Ser-Val-Ala
M 99	Arg-Lys-Ala-Ser-Val
M 126	Leu-Arg-Arg-Ala-Ser-Val
M 136	Ala-Arg-Thr-Lys-Arg-Ser-Gly-Ser-Val
M 144	Arg-Arg-Ala-Ser-Arg
M 157	Gva-Arg-Ala-Ser-Val
M 158	Leu-Arg-Arg-Ala-Ser-Val-Arg
M 160	Phe-Arg-Arg-Leu-Ser-Ile

technique. This will be further illustrated below with groups of even more closely related peptides associated with the phosphorylatable site of rat liver pyruvate kinase[14].

Fig. 2 shows the separation of three peptides, M 67, M 72 and M 73, all with *one* arginine residue. Components 2 and 3 contain the same amino acids. The only difference is that the two N-terminal amino acids appear in the reversed order, but even in this fast run all the peaks are well resolved. Furthermore, as shown in Fig. 3, four peptides, M 66, M 57, M 87 and M 97, all with *two* arginine residues, have been run and the corresponding peaks are well resolved. Starting from M 66, the four peptides form a series increasing by one neutral amino acid at a time up to the nonapeptide M 97. Although the difference between M 87 and M 97 is only one

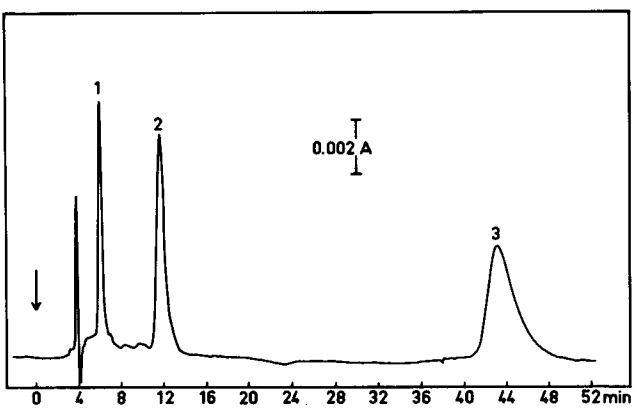


Fig. 1. Separation of three related basic peptides with one, two and three arginine residues, respectively. Mobile phase: phosphate buffer (pH = 3.1)–ethanol (79:21), flow-rate 1.3 mm/sec. Counter ion: hexanesulphonate (0.0105 *M*). Support: Spherisorb C_{18} (10 μm). Peaks: 1 = M 67; 2 = M 66; 3 = M 158 (for amino acid sequences see Table I).

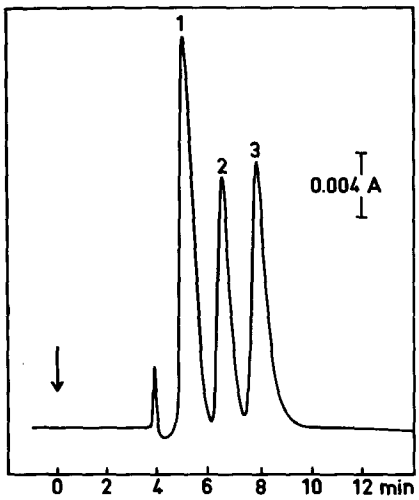
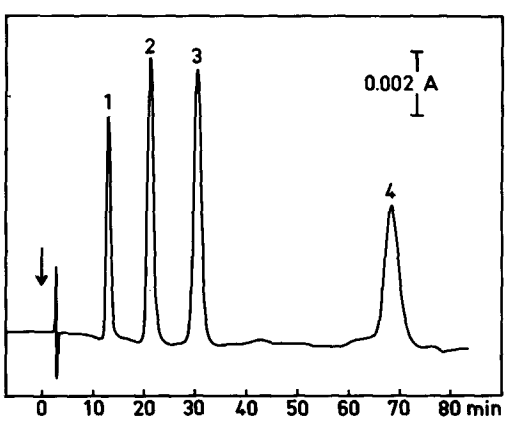


Fig. 2. Separation of closely related basic peptides all with one arginine residue. Mobile phase: phosphate buffer (pH = 4.5)–ethanol (72:28), flow-rate 1.3 mm/sec. Counter ion: hexanesulphonate (0.0094 *M*). Support: Spherisorb C_{18} (10 μm). Peaks: 1 = M 67; 2 = M 72; 3 = M 73 (for amino acid sequences see Table I).

glycine residue, the separation is considerable. Therefore, we do not anticipate any difficulties in expanding this series by at least a few more residues. In this context, however, we decided to proceed to peptides containing *three* arginine residues and Fig. 4 demonstrates the results obtained with two peptides of this type, M 144 and M



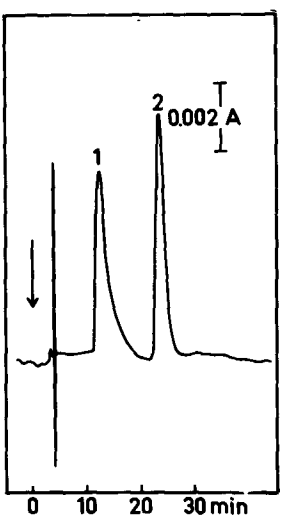


Fig. 3. Separation of four very closely related basic peptides all with two arginine residues. Mobile phase: phosphate buffer (pH = 4.5)–ethanol (79:21), flow-rate 1.3 mm/sec. Counter ion: hexanesulphonate (0.0105 *M*). Support: Spherisorb C_{18} (10 μm). Peaks: 1 = M 66; 2 = M 57; 3 = M 87; 4 = M 97 (for amino acid sequences see Table I).

Fig. 4. Separation of two very hydrophilic basic peptides with three arginine residues. Conditions as in Fig. 3. Peaks: 1 = M 144; 2 = M 158 (for amino acid sequences see Table I).

158. Even in this case the corresponding peaks are well resolved. Due to lack of material no peptides with four arginines have been run in our new system so far.

The influence of pH was preliminarily studied using the three pentapeptides, M 99, M 69 and M 73 (Fig. 5). The peptide M 99 differs from all previous peptides in that it contains a lysine residue in addition to an arginine. Peptide M 69 is the minimum substrate of cyclic AMP-stimulated protein kinase[2]. The three peptides elute from the column as three well separated peaks. This is particularly the case at pH 3.1.

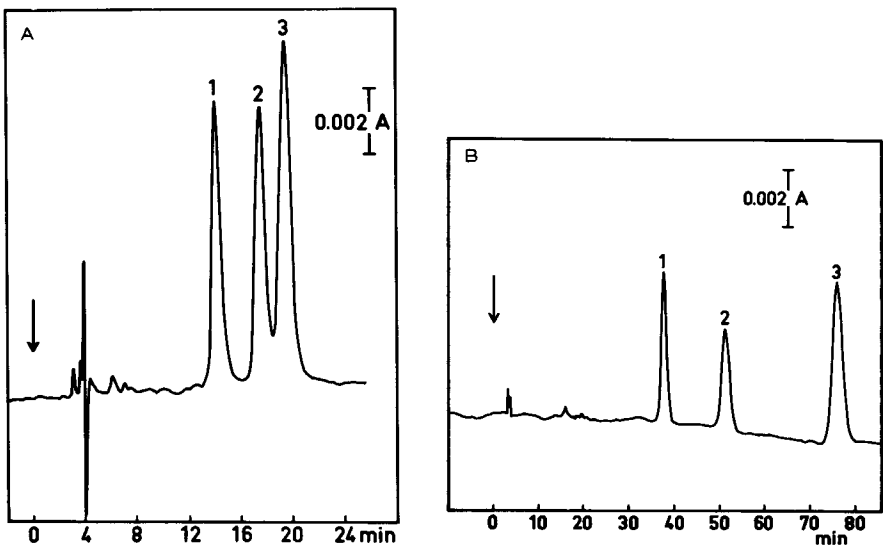
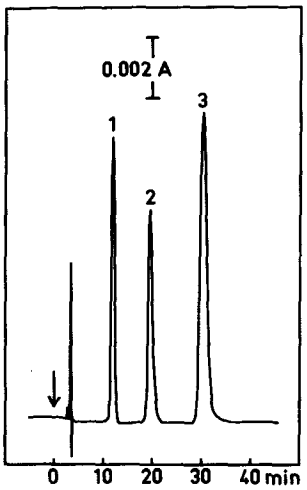


Fig. 5. Separation of three related hydrophilic peptides containing one or two basic amino acid residues. A, Mobile phase: phosphate buffer (pH = 4.5)–ethanol (75:25), flow-rate 1.3 mm/sec. Counter ion: hexanesulphonate (0.0113 *M*). Support: Spherisorb C_{18} (10 μm). Peaks: 1 = M 99; 2 = M 69; 3 = M 73 (for amino acid sequences see Table I). B, Mobile phase: phosphate buffer (pH = 3.1)–ethanol (75:25). Other conditions and samples as in A.

The influence of the α-amino group and a second hydrophobic amino acid is shown in Fig. 6 for peptides M 157, M 69 and M 126. The lower hydrophobicity of M 157 compared to M 69 is easily understood if we assume that M 157 can only bind two molecules of sulphonic acid[8], *i.e.*, one less than M 69. The addition of leucine to the N-terminus of M 69 has a similar effect upon the retention time of M 126 as seen from the comparison of M 66 and M 57 in Fig. 3.

Fig. 7 shows the separation of two peptides of different origins, M 136 and M 160. Peptide M 136 represents part of the phosphorylatable site of the β-subunit and M 160 that of the α-subunit of phosphorylase kinase[15]. These peptides are also substrates of cyclic AMP-stimulated protein kinase[5]. M 136 is an exceptionally hydrophilic peptide. This is clearly reflected in Fig. 7. On the other hand the occurrence of three hydrophobic amino acids in M 160 compared to only one in many of the previous peptides makes this peptide an interesting model compound for further studies.

As stated above our major interest has so far been to apply ion-pair HPLC to



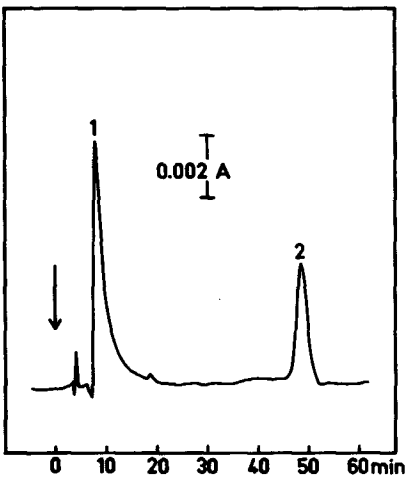


Fig. 6. Separation of closely related basic peptides. Mobile phase: phosphate buffer (pH = 3.2)–ethanol (72:28), flow-rate 1.3 mm/sec. Counter ion: hexanesulphonate (0.015 *M*). Support: Spherisorb C_{18} (10 μm). Peaks: 1 = M 157; 2 = M 69; 3 = M 126 (for amino acid sequences see Table I).

Fig. 7. Separation of two basic peptides related to the phosphorylatable site of the β-subunit of phosphorylase kinase and the α-subunit of the same enzyme. Mobile phase: phosphate buffer (pH = 3.1)–ethanol (79:21), flow-rate 1.3 mm/sec. Counter ion: hexanesulphonate (0.0105 *M*). Support: Spherisorb C_{18} (10 μm). Peaks: 1 = β-subunit peptide (M 136); 2 = α-subunit peptide (M 160; for amino acid sequences see Table I).

hydrophilic basic peptides. Nevertheless, as this work progressed we have applied the same system to analysis of a few other basic peptides like luteinizing hormone-releasing hormone (LHRH) and analogues of Substance P with promising results. Consequently we now plan a more systematic study of the effect of different separation parameters on resolution in order to improve further the systems described in this paper. For this reason a more detailed discussion of our present results from a theoretical and practical point of view will be postponed pending these experiments.

CONCLUSION

The purpose of this paper is to stress the usefulness of ion-pair HPLC in the analysis of basic hydrophilic peptides. Due to the influence of the hydrophobic counter ion hexanesulphonate, the basic functions are made less polar, and the peptides are more strongly bound to the reversed-phase support[8]. As a result, a number of basic, hydrophilic peptides, which otherwise would have been poorly retarded and resolved, were easily separated. Significant differences in retention time were seen upon variation of content of basic and hydrophobic amino acid residues, and even upon variation of the amino acid sequence.

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