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Separation of acidic protein tyrosine kinase substrates by strong anion-exchange chromatography

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Abstract

A series of heptapeptide sequences containing two glutamic and one aspartic acid residues, synthesized as substrates or inhibitors of different tyrosine kinases, and their phosphorylated or phosphonate analogs, were characterized analytically by strong anion-exchange high-performance liquid chromatography. Peptides of slightly different acidity can be separated under the experimental conditions used and the elution order increased in parallel with the number of acidic functions present in the sequence. The eluents and the characteristics of strong anion-exchange columns permit the scale-up of the method described to preparative purification.

Keywords: Peptides; Protein tyrosine kinases; Enzymes

1. Introduction

Synthetic peptides are largely employed in biochemical or biomedical research and their purification and characterization represent special chromatographic problems. In our laboratory, we have synthesized various peptides to study protein kinases and, in particular, the structural requirements by which these enzymes discriminate between different substrates [1,2]. At present, our research is addressed to study the biological properties of protein tyrosine kinases (PTKs) [2,3]. For this purpose, we synthesized the heptapeptide corresponding to the main autophosphorylation site of Src-PTK (EDNEYTA) and a great number of its analogs [3–5]. Such peptides can also be used in detecting and quantifying the activity of specific PTKs and in developing schemes for the isolation and purification of these enzymes. Among others, we synthesized analogs in which the tyrosine residue is phosphorylated (Tyr(P)) or substituted with an isosteric amino acid, such as 4-phosphonomethylphenylalanine (Pmp) or 4-phosphonophenylalanine (Pphe). These modified tyrosine derivatives were used as potential inhibitors of the phosphorylation process and also to study the properties of tyrosine phosphatases [6].

All of these peptides are characterized by the presence of a considerably high number of acidic residues, which induce a great decrease in the hydrophobicity. This occurrence is dramatically evident in the phospho and phosphono derivatives and causes several problems during HPLC purification procedures. The decrease in hydrophobicity is detrimental to peptide reversed-phase interactions and produces a loss of resolution. In addition, many of the contaminating species of crude synthetic peptides (truncated peptides, internal deletions, unremoved protecting groups, deamidated Gln and Asn residues,

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Table 1
Amino acid sequences of synthetic peptides and their apparent charges under the elution conditions

Peptides	Sequence	Charge
A	H–Glu–Asp–Asn–Glu–Tyr–Thr–Ala–OH	−3
B	Ac–Glu–Asp–Asn–Glu–Tyr–Thr–Ala–NH_2	−3
C	H–Glu–Asp–Asn–Glu–Tyr–Thr–Ala–NH_2	−2
D	*c*(Glu–Asp–Asn–Glu–Tyr–Thr–Ala)	−3
E	H–Glu–Asp–Asn–Glu–Tyr(P)–Thr–Ala–OH	−5
F	H–Glu–Asp–Asn–Glu–Pmp–Thr–Ala–OH	−5
G	H–Glu–Asp–Asn–Glu–Pphe–Thr–Ala–OH	−5

anisole adducts of Glu and Asp, and isopeptide bonds, among others) have very similar hydrophobicities to the expected products and, thus, may be resolved only with difficulty on reversed-phase (RP) columns.

In an early report [7], we demonstrated that ion pairing of unprotected carboxyl groups is a useful technique for the analysis of highly polar acidic peptides by RP-HPLC. The association of a peptide sample with a hydrophobic cation in the eluent leads to a less polar complex that has an increased retention time on RP columns. This approach, however, cannot easily be employed in preparative separations because of the difficulty of separating the counterions from the desired highly hydrophilic peptide by RP-HPLC, as reported when triethylammonium phosphate eluents were used [8]. Moreover, the high hydrophobicity induced by counterions in the ion-pair complexes [7] may mask the presence of contaminating species.

For these reasons, we explored new methods to analyze and purify our synthetic peptides. In this report, a simple and inexpensive method for the analysis of acidic peptides (Table 1) using strong anion-exchange (SAX) HPLC is described.

The results indicate that, depending on the anionic species involved, strong anion-exchange columns can be used for the analytical separation of this kind of peptide. Furthermore, the greater capacity of the ion-exchange matrix relative to the RP one, makes the former attractive also in the purification steps.

2. Experimental

2.1. Chemicals and reagents

All amino acid derivatives were prepared in our laboratory according to the procedure reported by Wünsch [9]. SAX-HPLC separations were performed using a linear A–B gradient elution technique, where A was 0.05% acetic acid (AcOH) in water and B was 0.05% trifluoroacetic acid (TFA) in water. In addition, the effect of an organic modifier was analyzed using elution systems containing different percentages of acetonitrile (MeCN). The HPLC-grade water was obtained from a Millipore Milli-Q apparatus (Bedford, MA, USA); HPLC-grade MeCN was obtained from Carlo-Erba (Milan, Italy).

2.2. Apparatus

The liquid chromatography system consisted of two LKB 2150 solvent delivery units (Uppsala, Sweden), an LKB 2152 solvent programmer and a 7125 Rheodyne injector (Cotati, CA, USA) coupled to a Knauer UV monitor (Berlin, Germany). The detection wavelength was 275 nm, for the aromatic ring, or 216 nm, for the peptide bond, according to the peptide being analyzed. The chromatogram was recorded on a C-R5A Chromatopac (Shimadzu, Kyoto, Japan).

2.3. Analytical separations

In a typical analytical experiment, 20 μl of a 1-mg/ml solution of peptide in water were injected. The pH of the samples was corrected to 7.5–8 with a NaOH solution. A Hypersil 5 μm SAX 300 Å, 250×4.6 mm column, obtained from Phenomenex (Torrance, CA, USA), was used. The separations were run at a nominal flow-rate of 1 ml/min at room temperature. The solvents used for anion-exchange

Table 2
Physicochemical properties of free heptapeptides

	Peptides	$[MH]^+$	Amino acid ratios						
			Xxx	Asp	Thr	Asn	Glu	Ala	Tyr
A	H–EDNEYTA–OH	841	–	1.98[a]	1.05	–	2.02	1.01	0.97
			–	0.97[b]	1.00	1.04	1.98	1.04	1.00
B	Ac–EDNEYTA–NH_2	882	–	1.99[a]	1.00	–	2.01	0.99	0.99
C	H–EDNEYTA–NH_2	840	–	2.00[a]	1.03	–	2.02	1.00	0.97
D	c(EDNEYTA)	823	–	1.97[a]	1.01	–	2.05	1.01	0.99
E	H–EDNE–pY–TA–OH	921	–[a]	1.98	0.98	–	2.04	1.00	1.00
			1.01[b]	1.00	0.98	1.04	2.08	0.95	–
F	H–EDNE–Pmp–TA–OH	919	1.02[a]	1.93	1.02	–	2.03	1.02	–
			0.99[b]	1.00	1.00	1.18	2.20	1.00	–
G	H–EDNE–Pphe–TA–OH	905	1.03[a]	1.99	0.96	–	2.06	0.99	–
			0.99[b]	1.00	0.91	1.03	2.10	0.96	–

Xxx=Tyr(P) or Pphe or Pmp.
[a]In acid hydrolysates.
[b]In enzymic hydrolysates.

chromatography were (A) 0.05% AcOH in water and (B) 0.05% TFA in water. In organic solvent influence experiments, mixtures of water and acetonitrile in different v/v ratios were used.

The hold-up time was determined by injection of an aqueous solution of acetone at the same nominal flow-rate, with detection at 320 nm.

2.4. Peptides

The peptides (Table 1) were prepared by a classical solution method using benzyloxycarbonyl–*tert*.-butyl (Z/tBu) strategy, with BOP-mediated [BOP, benzotriazol-1-yl-oxy-tris(dimethylamino)phosphonium hexafluorophosphate] couplings in tetrahydrofuran (THF). The Asn residue was incorporated as a dimethoxybenzhydryl (Mbh) derivative, while the Tyr residue was introduced with its side-chain function unprotected [3]. The phospho and phosphono analogs were prepared as previously described [6].

After application of convenient deprotection procedures (catalytic hydrogenolysis in the presence of 10% palladized charcoal and 98% aqueous TFA in the presence of 10% anisole, as scavenger), the final products were purified and the correct composition of the peptides was checked by determining the amino acid ratios in the acidic (6 *M* HCl, 110°C, 22 h) and enzymic hydrolysates and by laser desorption mass spectrometry (Table 2).

3. Results and discussion

Although various chromatographic systems containing TFA have been used successfully for the separation of many peptides on RP C_{18} columns, preliminary RP-HPLC experiments indicated that, even in the absence of an organic modifier, poor retention with a loss of resolution was exhibited by some of our highly acidic synthetic peptides. In order to increase the retention time of hydrophilic peptides, the use of cationic counterions for the separation of acidic substrates has been reported previously [7,10]. The extension of this approach to preparative separations is not easy, because counterions and salts present in the eluent can be removed only by chromatographic procedures, such as RP-HPLC [8]. This approach cannot be used for peptides that are either barely retained or not retained at all on RP-HPLC. In such cases, elution on an ion-exchange column, for example, is required. This additional step is not only tedious and sample-consuming, but is also a possible source of pollution.

For these reasons, we considered the possibility of eluting our peptides as anions on a SAX resin. In recent years, high-performance ion-exchange chromatography has become increasingly popular for the analysis and purification of peptides [11,12], both using anionic [13] and cationic [14,15] stationary phases. This chromatographic technique presents considerable disadvantages that are associated with the requirement of high salt concentrations to elute

the peptides and the presence of serious mixed-mode effects in many ion-exchange columns (particularly hydrophobic and ionic). The disadvantage of high salt concentrations, leaching and corrosion of stainless steel, as well as the incompatibility with certain analytical methods (physiological assays, mass spectrometric analysis, ion-exchange-based amino-acid analysis), were overcome using an elution method that includes changes in pH instead of ionic strength. The pH can affect the charge of the sample molecules and weaken or eliminate charge–charge interactions, thereby causing elution.

In consideration of these pH effects, for the purification and analysis of our acidic peptides, we used, as an elution system, a linear gradient between two different percentages of aqueous solutions of AcOH and TFA. In this way, we obtained a gradient from a pH value of 3.5 units to a value of 2.7 units. As a stationary phase, a strong anion-exchange resin was used. The advantage of using such a resin, as opposed to a weak anion-exchange column, lies in the fact that the pH of the mobile phase can be manipulated to change the net charge on the peptides without changing the properties of the stationary phase.

Care must be taken over the choice of ionic strength of the starting buffer (only 8 m*M* in the present case). High values of ionic strength can cause the elution of the peptides with unretained compounds.

Initially (Fig. 1), the chromatography was performed with a 3-min isocratic elution at 5% B and then a 40-min linear gradient from 5 to 40% B at a flow-rate of 1 ml/min (A was 0.05% AcOH in water; B was 0.05% TFA in water). From Fig. 1, it is clear that the peptides were eluted in order of increasing negative charge. Even the difference of a single charge results in a large change in retention time (i.e. **C** versus **A**, **B** and **D**). In addition, peptides that apparently had identical net negative charge were completely resolved (i.e. **A**, **B** and **D**, and **E**, **F** and **G**). This added selectivity has been attributed to the presence of a slight mixed-mode effect, additional to the primary ionic mode. Since peptides of identical

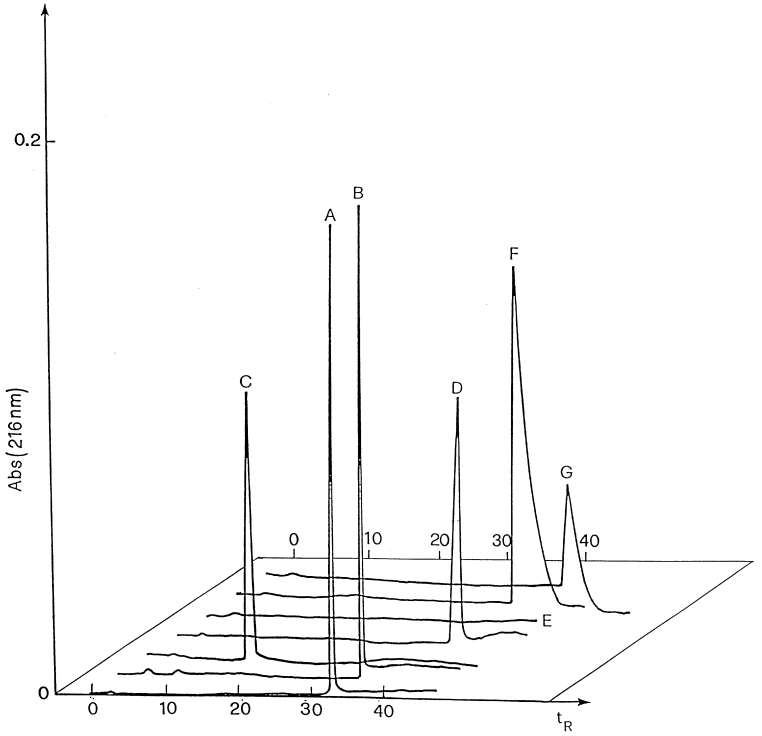

Fig. 1. Elution profile of synthetic peptides. Experimental conditions are reported in Section 2. Time is given in min.

charge are eluted in order of hydrophobicity, that is, the less hydrophobic peptides are eluted earlier than the more hydrophobic ones (see, for example, peptides **A**, **B** and **D**), the secondary mode can be ascribed to RP rather than normal phase interactions.

Subsequently, we examined the effect of an organic modifier, such as MeCN, in our experimental system. As reported in literature, the addition of organic solvents to the mobile phase affects different peptides in different ways, decreasing the retention time of some, while increasing the retention time of others [16]. In general, the organic solvent reduces the hydrophobic interactions between the molecules and the column packing surface and enhances solubility in some cases [17]. In practice, the optimal amount of organic solvent for the separation of a given peptide mixture must be determined empirically.

Also, in our experiments, the inclusion of MeCN in both mobile phases has a marked effect on selectivity in SAX-HPLC, as is apparent in Fig. 2. The addition of the organic solvent was found

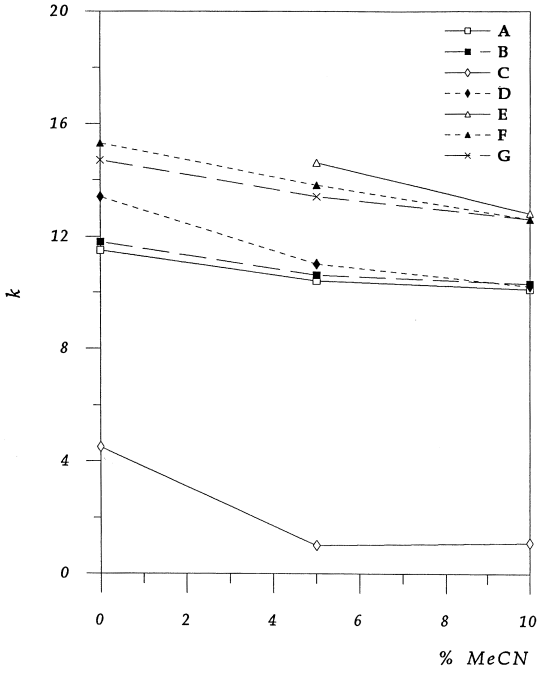

Fig. 2. Retention factor (k) values of synthetic peptides at increasing acetonitrile concentrations in the mobile phase. Experimental conditions are reported in Section 2.

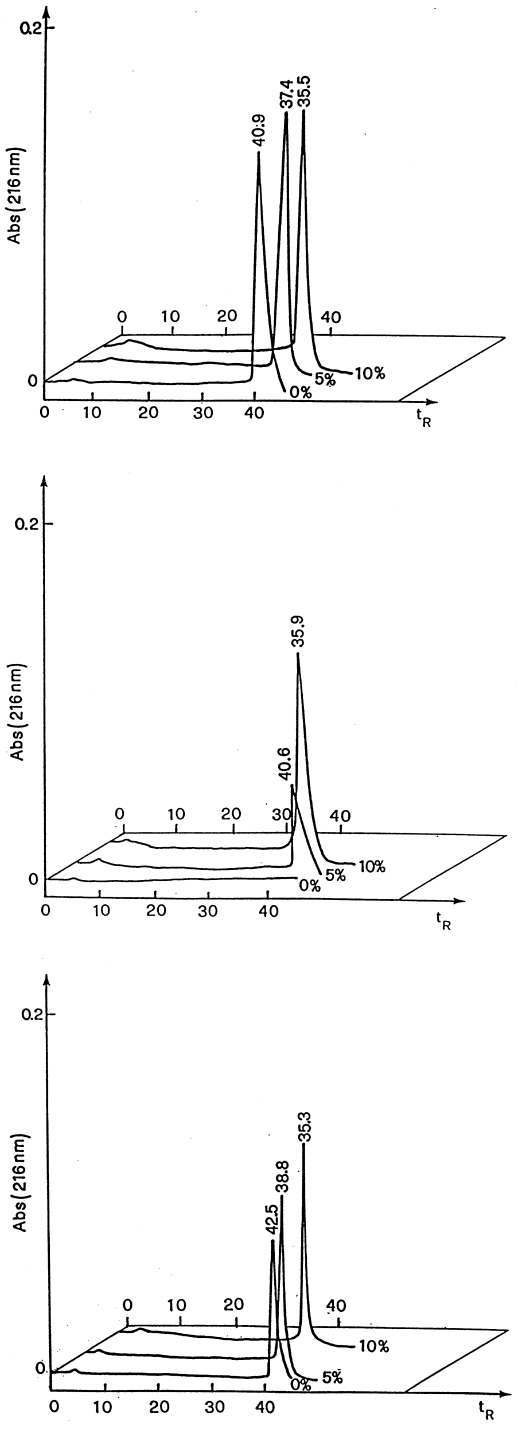

Fig. 3. Elution profiles of Pphe (top), Tyr(P) (middle) and Pmp (bottom) heptapeptides at increasing acetonitrile concentrations in the mobile phase. Experimental conditions are reported in Section 2. Time is given in min.

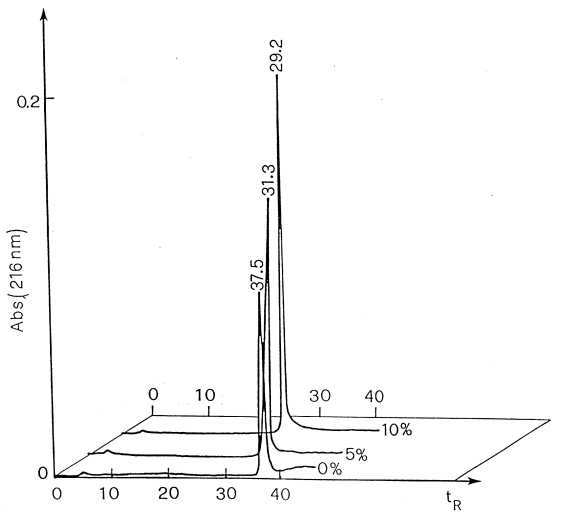

Fig. 4. Elution profiles of cyclic heptapeptide (**D**) at increasing acetonitrile concentrations in the mobile phase. Experimental conditions are reported in Section 2. Time is given in min.

necessary to elute the phospho (**E**) and phosphono peptides (**F** and **G**) within a reasonable time and with a reasonable peak shape (Fig. 3). In the absence of acetonitrile, the phosphotyrosine analog (**E**) was not eluted using the conditions reported, while phosphono and phosphonomethyl peptides were eluted as broad, skewed peaks. An increase in the MeCN concentration in the mobile phase also improved the peak shape of the cyclic analog (**D**) (Fig. 4). In general, retention times decreased with increasing MeCN concentration. The decrease in the *k* values for peptide **C** on changing the MeCN percentage from 0 to 5% was particularly evident (Fig. 2). However, the decrease of the retention times caused by the increasing presence of MeCN made the separation of peptides with the same net negative charge more difficult or impossible. A possible explanation of this effect may be that, in the absence of the organic solvent, the column packing was exhibiting hydrophobic, in addition to ionic, characteristics.

4. Conclusions

Anion-exchange chromatography should routinely be considered when designing peptide analysis and purification protocols. The relative retention times and the separation of peptides with identical charges at a given pH may be affected by changing the concentration of an organic modifier. Specifically, peptides with the same net charge but different hydrophobicity may be resolved.

A careful choice of the eluent permits, as reported in this paper, the use of an anion-exchanger as an alternative to reversed-phase peptide separation and the elimination of a desalting step after the ion-exchange column.

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