*Journal of Chromatography*, 548 (1991) 329–334
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2239

# Separation of acidic peptides by reversed-phase ion-pair chromatography

# Analytical application to a series of acidic substrates of casein kinases

A. CALDERAN*, P. RUZZA, O. MARIN, M. SECCHIERI, G. BORIN and F. MARCHIORI
*Biopolymer Research Centre of C.N.R., Department of Organic Chemistry of University of Padova, Via Marzolo 1, 35131 Padova (Italy)*

ABSTRACT

A series of small peptides including clusters of glutamyl residues, synthesized to study the site specificity of rat liver (L-CK2) and yeast (Y-CK2) casein kinase-2, are analytically characterized by ion-pair high-performance liquid chromatography using tetrabutylammonium as counter-ion and acetonitrile as modifier of the aqueous phase. Under these conditions peptides of slightly different acidity can be separated and the elution order parallels the hydrophobicity of the ion-pair–peptide complexes, which increases with the number of the acidic functions present in the sequence.

## INTRODUCTION

Synthetic peptides are largely employed as models to study various biochemical properties of protein kinases, in particular the mechanism by which these enzymes discriminate between different substrates and different potential phosphorylation sites. By varying the sequence of amino acid surrounding the target phosphorylated residue, it is in fact possible to detect the primary structure determinants that characterize the phosphorylation sites for a number of specific kinases. Such peptides can also be used in detecting and quantifying the activity of specific kinases in the presence of multiple kinases activities and in developing schemes for the isolation and characterization of these enzymes.

This approach was used in our laboratory to study the site specificity of two different casein kinases: the yeast casein kinase-2 (Y-CK2) and the rat liver casein kinase-2 (L-CK2). These enzymes catalyze the phosphorylation of a few specific seryl and threonyl residues in several proteins like casein. The phosphorylation sites in these proteins are always characterized by the presence of clusters of acidic amino acids on the C-terminal side of the target residue [1,2]. For this reason we have prepared, by the classical method in solution, a series of small peptides, including

0021-9673/91/$03.50 

clusters of glutamyl residues [3]. The structures of some of these peptides are given in Table I. They differ in the number and relative position of the acidic residues in the clusters, as well as in the nature of the target phosphorylatable residue.

In this paper a convenient method for the analytical separation of these peptides by high-performance liquid chromatography (HPLC) is reported.

## EXPERIMENTAL

### *Synthetic peptides*

The peptides used in this work are listed in Table I. The fragment condensation strategy in solution, which allows the utilization of several common intermediates for the preparation of the final peptides, was adopted. The standard procedures for peptide synthesis [4] have been employed, generally using the classical combination benzyloxycarbonyl and *tert.*-butyl groups for selective protection at the α-amino and γ-carboxyl functions, respectively. The methyl or ethyl esters, from which the corresponding hydrazides are readily obtained, were used to mask the α-carboxyl function in the intermediate peptides. The Rudinger modified azide procedure in the presence of N-hydroxysuccinimide as catalyst was therefore employed for fragment condensations, while mixed anhydrides (isobutyl chloroformate) or active esters (*p*-nitrophenyl of N-hydroxysuccinimido esters) were used for the preparation of the fragments.

After application of convenient deprotection procedures (catalytic hydrogenolysis in the presence of 10% palladized charcoal and/or exposure to 98% aqueous trifluoroacetic acid) the final peptides, when heterogeneous, were purified by chromatographic procedures (ionic exchange and gel filtration), then converted into hydrochloride salts by lyophilization with 5% HCl. The homogeneity of the final products was evaluated by thin-layer chromatography on cellulose plates and the correct composition of the peptides was checked by determining the amino acid ratios of the acid hydrolysates (6 *M* HCl, 110°C, 22 h) as previously reported [3].

TABLE I

AMINO ACID SEQUENCES OF THE SYNTHETIC PEPTIDES

| Peptide | Amino acid sequence |
|---|---|
| 1 | H–Ser–Glu–Glu–Glu–Glu–Glu–OH |
| 2 | H–Ser–Glu–Glu–Ala–Ala–Ala–OH |
| 3 | H–Ser–Ala–Ala–Glu–Glu–Glu–OH |
| 4 | H–Ser–Ala–Glu–Glu–Glu–Glu–OH |
| 5 | H–Ser–Glu–Ala–Glu–Glu–Glu–OH |
| 6 | H–Ser–Glu–Glu–Ala–Glu–Glu–OH |
| 7 | H–Ser–Glu–Glu–Glu–Ala–Glu–OH |
| 8 | H–Ser–Glu–Glu–Glu–Glu–Ala–OH |
| 9 | H–Thr–Glu–Glu–Glu–Glu–Glu–OH |
| 10 | H–Tyr–Glu–Glu–Glu–Glu–Glu–OH |
| 11 | H–Glu–Glu–Glu–Glu–Glu–Ser–$NH_2$ |
| 12 | H–Glu–Ser–Glu–Glu–Glu–Glu–Glu–OH |
| 13 | H–Ser–Ala–Glu–Glu–Glu–Glu–Glu–OH |
| 14 | H–Glu–Glu–Glu–Glu–Glu–OH |

*Chemicals*

All separations were performed using a linear A–B gradient elution technique, where A is $2 \cdot 10^{-2}$ *M* sodium phosphate buffer pH 5.6–$2 \cdot 10^{-3}$ *M* tetrabutylammonium hydrogensulfate. B is acetonitrile–$2 \cdot 10^{-3}$ *M* tetrabutylammonium hydrogensulfate. The solvent gradient was from 5 to 25% B in 35 min. HPLC-grade water was obtained from a Millipore Milli-Q apparatus (Millipore, Bedford, MA, USA); HPLC-grade acetonitrile was obtained from Farmitalia-Carlo Erba (Milan, Italy); tetrabutylammonium hydrogensulfate and sodium dihydrogenphosphate dihydrate were obtained from Fluka (Buchs, Switzerland) and were of analytical grade.

*Equipment*

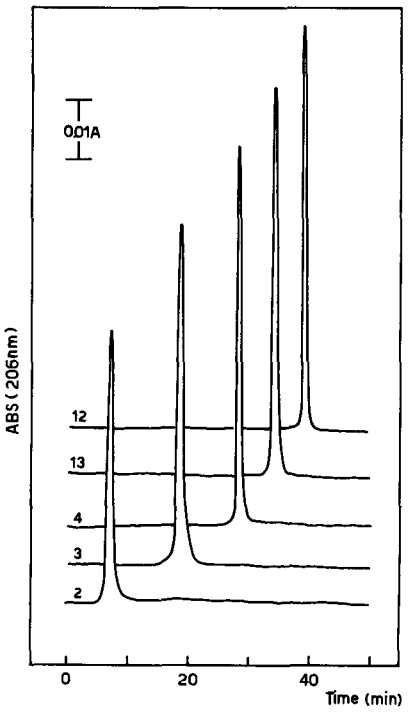
The liquid chromatographic system consisted of two LKB 2150 solvent delivery units (Pharmacia LKB Biotechnology, Uppsala, Sweden), a LKB 2152 solvent programmer and a 7125 Rheodyne injector (Rheodyne, Cotati, CA, USA) coupled to a LKB 2158 UV monitor. The detection wavelength was 206 nm. In a typical experiment, approximately 10 nmol of each peptide, dissolved in 15 $\mu$l of water containing 0.05% 1 *M* NaOH, was injected. The separation column was a ROSil $C_{18}$, 3 $\mu$m (100 × 4.6 mm I.D.), always preceded by a short pre-column (Alltech Assoc., Deerfield, IL, USA). The separations were run at a nominal flow-rate of 1 ml/min at room temperature (22°C) and the pH measurements were performed at the same temperature.

## RESULTS AND DISCUSSION

The analytical separation of slightly different peptides requires often difficult and time-consuming procedures. With our synthetic glutamic acid-containing peptides (Table I), the presence of one or more acid groups can generally be exploited for separation by ion-exchange chromatography, but acceptable results are obtained only with a long elution times. On the other hand, very good separations are possible using another chromatographic approach: the generation of hydrophobic ion-pairs of these peptides [5]. In this procedure, the acidic peptides are associated with a hydrophobic cation in the eluent leading to less polar complexes [6,7]. Obviously the hydrophobicity of these ion-pair complexes and consequently their retention times on a reversed-phase column increase with the number of acidic functions. As described in Experimental section, the ion-pairing reagent used with success in the water–acetonitrile mobile phase is the hydrophobic tetrabutylammonium ion. The corresponding separation patterns are shown in Figs. 1, 2 and 3.

Fig. 1 shows the separation of the amino- and carboxy-terminal free peptides 2, 3, 4, 13 and 12. From a structural point of view they can be considered sufficiently similar, but possess an increasing number of glutamyl residues: two, three, four, five and six, respectively. In agreement with the prediction, the elution patterns evidence that in the presence of tetrabutylammonium hydrogensulfate the hydrophobicity of the ion-pair complexes increases with the number of acidic groups. In particular, the resulting differences in retention time are considerable and give a clear indication of the usefulness of this chromatographic system.

In Fig. 2 the separation patterns of the six peptides (1, 9, 10, 11, 13 and 14) containing five glutamic acid residues are shown. The similarity in the retention times of compounds 1, 9, 10 and 14, which differ only in the side-chain of the N-terminal



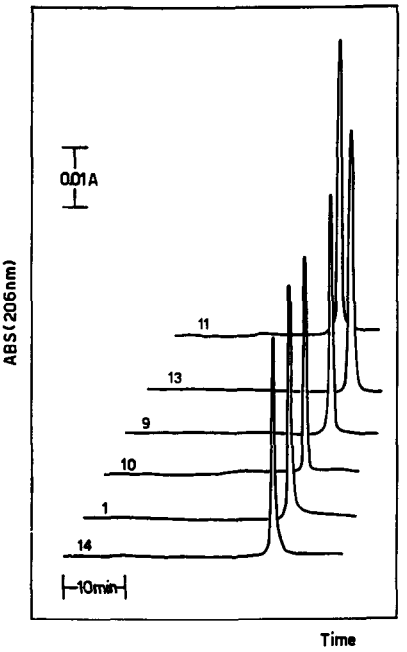




Fig. 1. Elution profiles of five peptides with an increasing number (2–6) of glutamyl residues. The amino acid sequences (one-letter codes) and the corresponding retention times are : 2 = SEEAAA (6.5 min); 3 = SAAEEE (17.5 min); 4 = SAEEEE (27.0 min); 13 = SAEEEEE (33.0 min); 12 = ESEEEEE (37.0 min). Experimental conditions as reported in *Chemicals* and in *Equipment*.

Fig. 2. Elution profiles of six peptides all containing five glutamic acid residues. The amino acid sequences and the corresponding retention times are: 14 = EEEEE (34.0 min); 1 = SEEEEE (33.5 min); 10 = YEEEEE (33.0 min); 9 = TEEEEE (33.5 min); 13 = SAEEEEE (33.0 min); 11 = EEEEES-amide (27.5 min). Experimental conditions as reported in *Chemicals* and in *Equipment*.

residue (serine, threonine, tyrosine or absent, respectively) indicates that a difference in the hydrophobicity of only one residue does not induce a substantial change in the elution profile of the ion-pair complexes. One additional residue, alanine, causes little change in the chromatographic behaviour of the five glutamyl residues containing peptides, *e.g.* compare compounds 13 and 1. Considering the results of Fig. 1 also, we can conclude that the influence of a change of the nature of residue(s) in the backbone seems to be very limited, especially in the presence of a high number of acidic functions. A direct comparison of the influence of the presence of a seryl residue in the N- or C-terminal position between compounds 1 and 11 is obviously impossible due to the amidation of the C-terminal carboxylic function in compound 11. Notwithstanding this, the corresponding consistent drop in the retention time of the ion-pair complex of 11 even if predicted, again confirms the high sensitivity of this chromatographic system.

In addition, Fig. 3 demonstrates the limited influence of the order in which the amino acid residues are arranged in the sequence. In fact the retention times reported

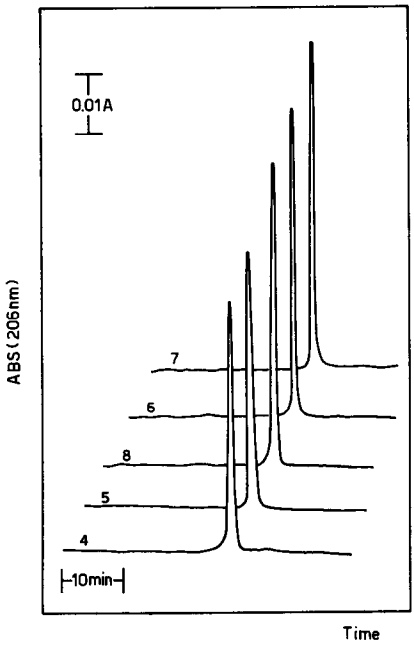



Fig. 3. Elution profiles of five hexapeptides all with the identical amino acid ratios $Ser_1Glu_4Ala_1$. The amino acid sequences and the corresponding retention times are: 4 = SAEEEE (27.0 min); 5 = SEAEEE (26.5 min); 8 = SEEEEA (27.5 min); 6 = SEEAEE (26.5 min); 7 = SEEEAE (26.5 min). Experimental conditions as reported in *Chemicals* and in *Equipment*.

in this figure for the five hexapeptides 4, 5, 6, 7 and 8, all having identical amino acids ratios ($Ser_1Glu_4Ala_1$) but different sequential arrangement, are almost coincident. Specificcally, no effect on the chromatographic behaviour is detectable for peptides containing acidic clusters of different length if the total number of acidic functions is the same.

## CONCLUSIONS

The goal of this paper is to demonstrate that reversed-phase HPLC using ion-pairing reagents previously applied to the analysis of basic hydrophilic peptides [8,9] can also be usefully employed in the analytical characterization of acidic hydrophilic peptides. In fact, using a reversed-phase support to which the tetrabutylammonium ion-pair–peptide complexes are strongly bound, it is possible to separate ion-pair–peptide complexes differing in the number of the acidic functions. The differences in the retetion times are sufficient to allow the separation of peptides differing in only one acidic function. On the contrary, the high hydrophobicity induced by the tetra-butylammonium ion in the ion-pair–peptide complexes may mask the variations in the amino acid sequence, particularly when a high number of acidic functions is present.

ACKNOWLEDGEMENTS

This work was supported by grants from the Italian 'Ministero della Pubblica Istruzione' and 'Consiglio Nazionale delle Recerche' (Progetto Finalizzato 'Chimica Fine e Secondaria'). The amino acid analyses were carried out by Ugo Anselmi and Ernesto De Menego. The skilful technical assistance of Luca Bani in the synthetis of compounds is gratefully acknowledged.

REFERENCES

1 F. Meggio, A. P. Boulton, F. Marchiori, G. Borin, D. P. W. Lennon, A. Calderan and L. A. Pinna, *Eur. J. Biochem.*, 177 (1988) 281–284; and references cited therein.
2 F. Marchiori, F. Meggio, O. Marin, G. Borin, A. Calderan, P. Ruzza and L. A. Pinna, *Biochim. Biophys. Acta*, 971 (1988) 332–338; and references cited therein.
3 O. Marin, A. Calderan, P. Ruzza, G. Borin, F. Meggio, N. Grankowsky and F. Marchiori, *Int. J. Pept. Prot. Res.*, 36 (1990) 374–380.
4 E. Wünsch, in E. Wünsch (Editor), *Houben-Weyl, Methoden der Organischen Chemie*, Vol. 15, Georg-Thieme Verlag, Stuttgart, 1974.
5 E. Tomlinson, T. M. Jefferies and C. M. Riley, *J. Chromatogr.*, 159 (1978) 315–358.
6 W. S. Hanckock, C. A. Bishop, J. E. Battersby, D. R. K. Harding and M. T. W. Hearn, *J. Chromatogr.*, 168 (1979) 377–384.
7 D. Guo, C. T. Mant and R. S. Hodges, *J. Chromatogr.*, 386 (1987) 205–222.
8 B. Fransson, U. Ragnarsson and Ö. Zetterqvist, *J. Chromatogr.*, 240 (1982) 165–171.
9 B. Fransson, U. Ragnarsson and Ö Zetterqvist, *Anal. Biochem.*, 126 (1982) 174–178.