Journal of Chromatography, 535 (1990) 33–39
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2070

Post-column continuous-flow analysis combined with reversed-phase liquid chromatography and computer-aided detection for the characterisation of peptides

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ABSTRACT

Peptide mapping is a key technique for structural identification of new proteins or the products of recombinant gene technology. The recognition of oligopeptides, separated by reversed-phase liquid chromatography, is limited by the conventional reliance on the correlation of retention times with standards, supported by dual-wavelength chromatograms. It has been reported that the recognition of phenolic compounds can be achieved by a novel technique, based on computer-aided photodiode-array detection of the pH-shifted solutes after post-column continuous-flow analysis. This work describes how the generation of the pH-shifted difference spectra for dipeptides, containing a tyrosyl residue, may be used to enhance peak recognition, when used in conjunction with absorbance ratios.

INTRODUCTION

Combination of liquid chromatography photodiode-array detection (PDAD) with flow-injection analysis (FIA) as a post-column reaction system offers several possibilities for enhanced detection capability. Recognition of phenolic compounds can be achieved by a novel technique, based on computer-aided PDAD of the pH-shifted solutes after post-column continuous-flow analysis [1,2]. It is proposed that this technique may be used in the characterisation of peptides, such as those generated from tryptic digests. Although phenylalanine (Phe), tyrosine (Tyr), and tryptophan (Trp) display characteristic chromophores above 230 nm, which allow characterisation by PDAD [3], the extensive band overlap leads to complex composite profiles in peptide mixtures. Moreover, in samples of biological origin, additional absorbing constituents often contribute spectral interference. A common problem arises from the closely overlapping chromophores of Tyr and Trp between 270 and 280 nm. Although Trp, being rather more absorptive than Tyr, tends to dominate the spectrum, the labile phenolic group of Tyr permits its spectrum to be shifted to higher wavelength (*ca.* 293

0021-9673/90/$03.50

nm) by raising the solution pH. Since the Trp chromophore is less pH-sensitive, this feature may form the basis for resolving peptides containing varying proportions of these amino acids. The use of pH shifting as a peak recognition technique, for various dipeptides containing a tyrosyl residue, has been investigated and is reported below.

EXPERIMENTAL

Reagents

Methanol [high-performance liquid chromatographic (HPLC) grade, Rathburn, Walkerburn, U.K.] was used as received. The 5-m*M* potassium dihydrogenphosphate solution (AnalaR, BDH, Poole, U.K.) and 0.1 *M* potassium hydroxide solution (Convol, BDH, Poole U.K.) were prepared with glass-distilled water and filtered through 0.45-μm filters (Millipore Waters, Middlesex, U.K.), using all-glass equipment. All eluents and FIA carrier streams were degassed for 10 min in an ultrasonic bath under reduced pressure. L-tyrosine (Tyr), L-tyrosyl-L-tyrosine (Tyr–Tyr), L-tyrosylglycine (Tyr–Gly), glycyl-L-tyrosine (Gly–Tyr), L-tyrosyl-L-phenylalanine (Tyr–Phe), L-phenylalanyl-L-tyrosine (Phe–Tyr), tryptophyl-tyrosine (Trp–Tyr) and L-phenylalanyl-glycine (Phe–Gly) were obtained from (Sigma, St. Louis, MO, U.S.A.). Solutions of the dipeptides and tyrosine (all *ca.* 250 μM) were prepared in distilled water.

Apparatus

The apparatus was configured as shown in Fig. 1 [3]. The chromatographic system used consisted of a Kratos SF-400 pump (Kratos Analytical, Warrington, U.K.) with a rheodyne injection valve (Model 7125) provided with a 20-μl loop, together with an ABI 1000S diode-array detector (Applied Biosystems (ABI) Ramsey, NJ, U.S.A.) and a BD-40 chart recorder (Kipp & Zonen, Delft, The Netherlands). The ABI 1000S was interfaced, via a RS-232C link, to an IBM-compatible personal computer (Elonex, Bradford, U.K.) with a Panasonic KX-P1081 printer (supplied by ABI) and a HP-ColorPro plotter (Hewlett-Packard, Cheadle Heath, Stockport, U.K.). The data acquisition and manipulation was performed using Lab. Calc. software (Galactic Industries, Salem, NH, U.S.A.). Spectra acquired, in the range 230–350 nm, during chromatography were normalised with reference to maximal absorbance at the λ_{max}, using the Lab. Calc. facility to apply the appropriate scale multiplier. This

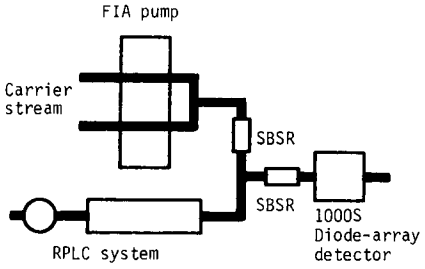

Fig. 1. Experimental system combining liquid chromatography with post-column continuous-flow analysis and photodiode-array detection.

enabled the difference spectrum to be obtained in such a way that any changes in the intrinsic profile of the spectrum can be obtained in a standardised format.

The post-column reaction system consisted of a Gilson Minipuls-3 peristaltic pump (Anachem, Luton, U.K.), together with Elkay accu-rated tubing (Laboratory Products, Basingstoke, U.K.) and two single-bead string reactors (SBSRs) (100 cm × 0.8 mm I.D. and 50 cm × 0.8 mm I.D.) to promote mixing. All connections were made with 0.5 mm I.D. PTFE tubing. The FIA carrier stream was combined with the post-column eluent stream by a suitable T-piece (Anachem). The two FIA carrier streams were combined through a suitable Y-piece (Anachem).

LC–FIA conditions

A stainless-steel column (250 mm × 4.6 mm I.D.) packed with Techsphere 5 μm ODS (HPLC Technology, Macclesfield, U.K.) was used. The mobile phase, pumped at 1.0 ml/min, consisted of, for Tyr, Gly–Tyr and Tyr–Gly, methanol–5 m*M* KH_2PO_4 (pH 4) buffer (10:90, v/v) and for Tyr–Phe, Phe–Tyr, Phe–Gly, Tyr–Tyr and Trp–Tyr, methanol–5 m*M* KH_2PO_4 (pH 4) buffer (15:85, v/v). Detection was effected using the diode-array at 272 nm. The FIA system consisted of 0.03 ml/m tubing, the pump speed was set to deliver 0.05 ml/min from each of the two FIA carrier streams. 0.1 *M* KOH was used to effect the post-column pH-shift.

RESULTS AND DISCUSSION

The pH-shifted difference spectrum was obtained for each dipeptide by subtracting the normalised spectrum under alkaline conditions (apparent pH of the methanolic mobile phase, pH* 12.4) from the spectrum obtained under the reversed-phase liquid chromatography (RPLC) conditions (pH* 4.4) as illustrated with Gly–Tyr in Fig. 2. As previously reported, the mobile phase and potassium hydroxide solution concentrations used were such that they had similar spectra [4]. Data from wavelengths below 230 nm were not used because solvent effects, due to absorption by alkali, were observed even in the spectrum of Phe–Gly. A comparison of the difference between the spectra of the normalised dipeptides and the normalised pH-shifted difference spectra of the dipeptides is shown in Fig. 3. Visual examination of these data reveals no obvious advantages in the use of pH-shifted difference spectra to enhance recognition.

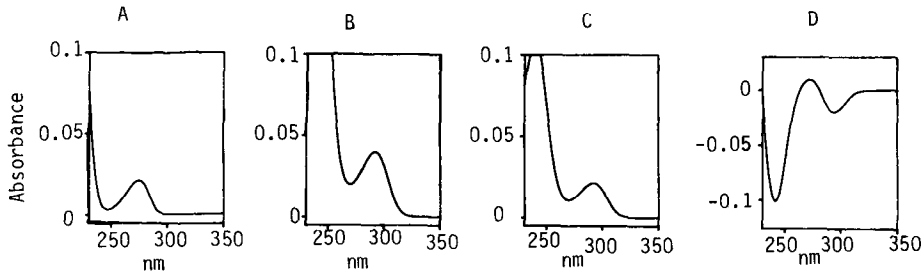

Fig. 2. Spectral manipulation to obtain the pH-shifted difference spectrum. (A) Gly–Tyr, pH* 4.4; (B) Gly–Tyr, pH* 12.4; (C) Gly–Tyr, pH* 12.4, normalised; (D) Gly–Tyr, difference spctrum.

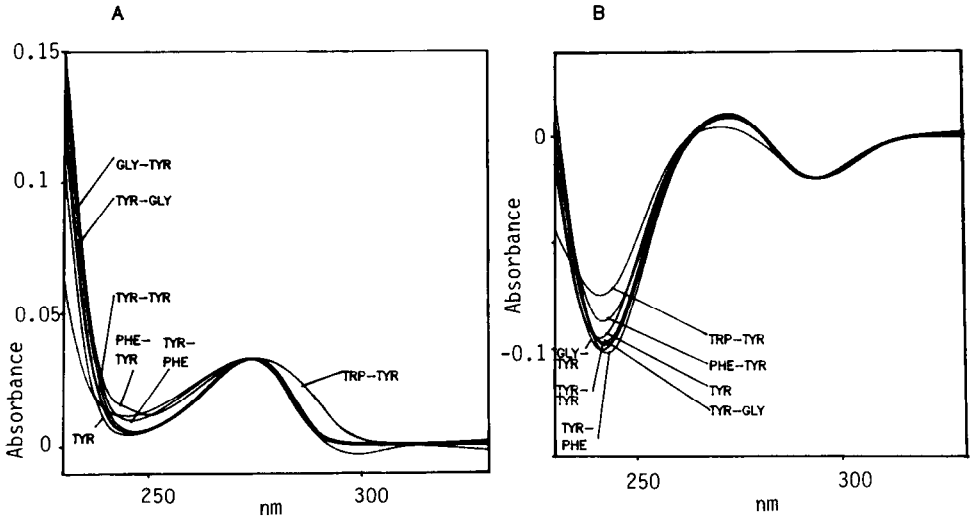

Fig. 3. A comparison of the difference between the normalised spectra (A) and the normalised pH-shifted difference spectra (B) of the dipeptides.

The use of absorbance ratios has been reported as a powerful technique for solute identification and sample discrimination [5]. This technique was investigated for the recognition of the dipeptides by both spectral and pH-shifted difference spectral data. To determine the wavelengths of choice for good discrimination between the peptides, the difference spectrum between normalised difference spectra of the two

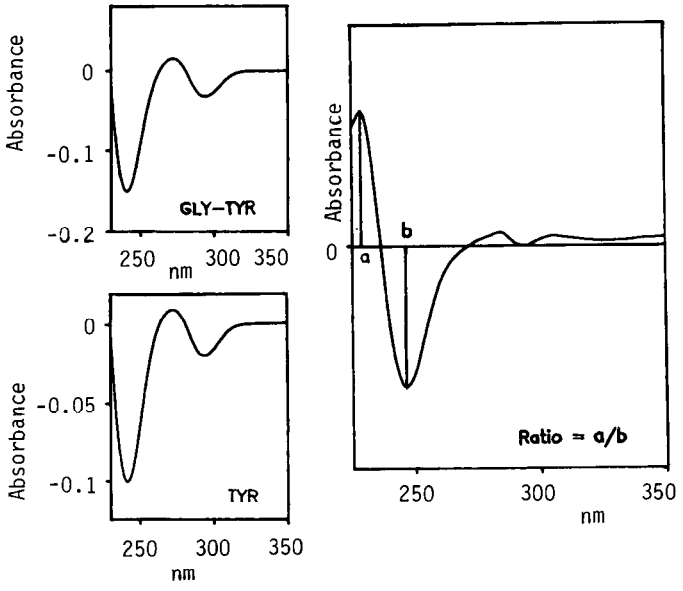

Fig. 4. Determination of the wavelength for the absorbance ratio of choice, for good discrimination between the peptides. Right: the difference spectrum between normalised difference spectra of Gly–Tyr and Tyr.

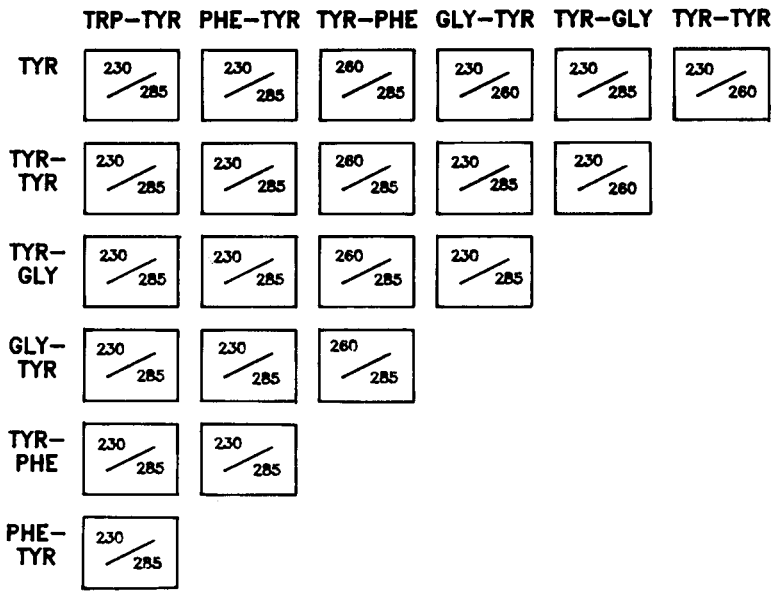

Fig. 5. The modified absorbance ratio wavelengths of choice for discrimination between dipeptides containing a Tyr residue: unshifted spectra.

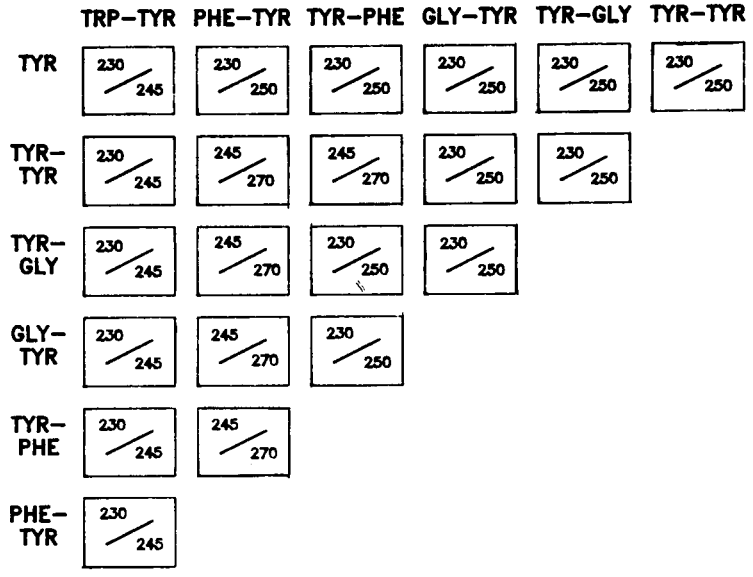

Fig. 6. The modified absorbance ratio wavelengths of choice for discrimination between dipeptides containing a Tyr residue: pH-shifted difference spectra.

peptides was computed. The wavelengths of maximum positive and negative Δ absorbance (above 230 nm for difference spectra) were recorded. This is illustrated by Fig. 4.

To facilitate automation of this technique, the number of wavelengths for the absorbance ratios was reduced, to establish six ratios for use with both the unshifted and pH-shifted difference spectra. In most cases, this necessitated a change of less than 3 nm from the wavelength determined above, and in all cases a significant difference was observed between the two normalised spectra at the chosen wavelengths. Figs. 5 and 6 show the simplified absorbance ratios.

The absorbance ratios calculated from the data determined above are shown in Tables I and II.

It can be observed, by comparing Figs. 1 and 2 with the results obtained (Tables I and II) that the method used for determination of the wavelengths of choice for the absorbance ratios did not always give the greatest numerical difference between the dipeptides in question, hence further work is being undertaken to improve the method

TABLE I

ABSORBANCE RATIOS FOR PEAK RECOGNITION OF UNSHIFTED SPECTRA

x = mean, R.S.D. = % relative standard deviation, n = 9.

Ratio	x (R.S.D.)		
	285/230 nm	260/230 nm	285/260 nm
Tyr	0.138 (0.43)	0.156 (0.23)	0.882 (0.27)
Tyr–Tyr	0.112 (0.69)	0.119 (0.33)	0.944 (0.38)
Tyr–Gly	0.109 (0.54)	0.134 (1.59)	0.815 (0.93)
Gly–Tyr	0.134 (1.09)	0.134 (1.54)	1.00 (0.47)
Tyr–Phe	0.091 (6.64)	0.147 (4.85)	0.620 (2.32)
Phe–Tyr	0.106 (2.08)	0.138 (2.70)	0.766 (0.78)
Trp–Tyr	0.405 (0.08)	0.367 (0.17)	1.10 (0.15)

TABLE II

ABSORBANCE RATIOS FOR PEAK RECOGNITION OF pH-SHIFTED DIFFERENCE SPECTRA

x = mean, R.S.D. = % relative standard deviation, n = 9.

Ratio	x (R.S.D.)		
	230/250 nm	270/245 nm	230/245 nm
Tyr	0.223 (0.67)	−0.108 (0.30)	0.152 (0.72)
Tyr–Tyr	−0.088 (10.7)	−0.088 (2.52)	−0.064 (10.9)
Tyr–Gly	−0.081 (27.0)	−0.101 (2.86)	−0.059 (27.1)
Gly–Tyr	0.080 (6.43)	−0.097 (0.31)	0.056 (6.39)
Tyr–Phe	−0.260 (14.0)	−0.099 (4.64)	−0.193 (13.7)
Phe–Tyr	−0.128 (12.5)	−0.093 (5.81)	−0.093 (12.2)
Trp–Tyr	0.909 (1.71)	−0.058 (4.56)	0.641 (1.65)

for determining absorbance ratios so that sensitivity may be improved. In this respect, the method of multiple absorbance ratio correlation as proposed by Marr *et al.* [6] has potentially interesting applications for peptide recognition.

Examination of the absorbance ratios for the characterisation of the unshifted spectra shows that, with the exception of Trp–Tyr, the dipeptides have similar ratios. Comparison of the ratios generated from the pH-shifted difference spectra reveals that, in general, greater differences are observed between the dipeptides. This is partly a result of both positive and negative ratios being generated from the difference spectra. The greater relative standard deviations observed for the pH-shifted difference spectra may be attributed to the increased background noise created by this technique. Despite this increased variation, the ratios generated are sufficiently different, for the dipeptides, for this technique to be a useful addition to the tools available in peptide recognition.

It has previously been reported that the relative standard deviation for absorbance ratios is dependent on the absorbance at the wavelengths of interest, in addition to background noise [5]. Hence, it is unlikely that the technique as proposed could be used in the characterisation of sub-nanogram quantities of dipeptides, on column.

CONCLUSIONS

The generation of the pH-shifted difference spectra for dipeptides containing a tyrosyl residue, may be used to enhance peak recognition, when used in conjunction with absorbance ratios. It is proposed that this low cost technique may be used to enhance the characterisation of oligopeptides generated by tryptic mapping. The use of absorbance ratios common to various peptides will allow automation of the technique for the analyst.

ACKNOWLEDGEMENTS

The authors thank the British Council and the Spanish Government for supporting part of this work under the Acciones Integradas Programme. Valuable discussions with Professor M. Valcarcel and M.D. Luque De Castro are acknowledged with thanks. One of the authors (J.B.C.) would like to thank Pharmacia Leo Therapeutics AB for providing the studentship for this research.

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