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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND DIODE-ARRAY DETECTION FOR THE IDENTIFICATION OF PEPTIDES CONTAINING AROMATIC AMINO ACIDS IN STUDIES OF ENDORPHIN-DEGRADING ACTIVITY IN HUMAN CEREBROSPINAL FLUID

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SUMMARY

Diode-array UV detection has been adapted for analysis of opioid peptides and their metabolic fragments differing in aromatic amino acid content. In combination with high-performance liquid chromatography, the technique allowed a direct and rapid discrimination between peptides containing phenylalanine, tryptophan and tyrosine, or a combination of these residues. Enkephalin fragments with either tyrorosine or phenylalanine, or both, were identified after digestion of the pentapeptide with proteolytic activity recovered from human cerebrospinal fluid. The N-terminal tyrosine-containing fragment of dynorphin A was identified after hydrolysis of the peptide by a cerebrospinal fluid endopeptidase. The study was extended to the analysis of some non-opioid peptides. The Tyr[1] analogue of delta-sleep-inducing peptide was easily distinguished from the authentic compound with a tryptophan at the N-terminus. Results indicated that the technique was useful for discriminating between dipeptides differing in aromatic residues that were unresolved by high-performance liquid chromatography.

INTRODUCTION

Photodiode-array UV detection was recently introduced in the high-performance liquid chromatography (HPLC) of aromatic amino acids, their metabolites, and dipeptides containing aromatic residues[1]. This detection technique allows acquisition of spectral information at sequential time intervals under computer control during elution. It also provides a high potential for manipulation of recorded chromatographic data. By computer-aided graphics, absorption spectra of separated peaks can rapidly be compared by means of two- or three-dimensional visualization. In the present study, focused on endorphins and other neuropeptides, computer-controlled diode-array detection was used for (1) the identification of peptides containing aromatic amino acids; (2) discriminating spectral analyses to discern peptides containing

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tryptophan, tyrosine and phenylalanine, or a combination of these residues; and (3) the determination of peak purity.

Neuropeptides participate in many events of the nervous and humoral system. They represent a class of highly active substances, for which efficient inactivation mechanisms are likely to exist. During the last decade, a number of enzymes capable of degrading these peptides have been identified in various tissues[2]. In recent studies, several neuropeptide-degrading enzymes have been observed in cerebrospinal fluid (CSF)[3–6]. Among these are, *e.g.*, the angiotensin-converting enzyme (ACE)[3] and enkephalinase[4], known to hydrolyse substance P and the enkephalins. Since CSF is in direct contact with the central nervous system (CNS), its proteolytic activity is of particular interest. (Met)- and (Leu)enkephalin (Tyr-Gly-Gly-Phe-Met or -Leu), the endogenous opioid pentapeptides, and the C-terminally extended analogue dynorphin (Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asn-Asp-Glu) are believed to play an important role in neurotransmission. After their release and action, they are rapidly degraded, most likely by specific enzyme systems. Several peptidases capable of hydrolysing the enkephalins are known, but to understand the mechanism of their degradation fully, further studies are needed. This presentation describes the use of HPLC and diode-array detection to monitor the degradation of (Leu)enkephalin and dynorphin by proteolytic activities isolated from human CSF. The equipment was also used to analyse the tryptophan-containing delta-sleep-inducing peptide (DSIP; Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu) and some dipeptides differing in aromatic amino acid residues.

MATERIALS AND METHODS

Peptides and chemicals

Reference peptides were purchased from Bachem (Bubendorf, Switzerland). The chromatographic media Sephadex G-100 and DEAE-Sepharose CL-6B were from Pharmacia Fine Chemicals (Uppsala, Sweden). The protease inhibitor, Captopril was obtained from Squibb (Princeton, NJ, U.S.A.). All other chemicals and solvents were of analytical-reagent grade from various commercial sources.

Enzyme preparation

The enzyme fraction used in this study was obtained from a side-fraction described in a previously published procedure for the partial purification of a substance P-converting endopeptidase from human CSF[5]. Thus, the purification involved ion-exchange chromatography (DEAE-Sepharose CL-6B) and gel chromatography (Sephadex G-100). Fractions eluted in the molecular weight (MW) range of around 40 000 were collected for purification of the substance P-converting activity, whereas those eluted ahead of human serum albumin (MW 68 000) were lyophilized and used as enzyme sources in the present study. Fractions eluted in this area of the chromatogram have previously been shown to contain ACE and enkephalinase-like activity[6,7].

Another CSF enzyme used in this study is capable of releasing the N-terminal (Leu)enkephalin-Arg[6] sequence from dynorphin and alpha-neoendorphin. Its purification was described in a recent paper[8].

Enzyme incubation

The freeze-dried enzyme fraction was reconstituted in 20 m*M* Tris–HCl buffer (pH 7.8) to give a protein concentration of 2 mg/ml. Aliquots of 20 μl were incubated with 30 μg of substrate in a final volume of 50 μl of the above buffer. In the experiments with the ACE and enkephalinase-like activity, Captopril (10 μM) was added to the incubation vial. In experiments with the dynorphin-converting endopeptidase, no inhibitor was added. The incubations were performed at 37°C and allowed to proceed overnight. Reactions were terminated by boiling the incubate for 1–2 min before the addition of 1 ml of methanol and removal of protein by centrifugation (1 min with a Beckman Microfuge B, Beckman Instruments, Palo Alto, CA, U.S.A.). The samples were subsequently taken to dryness (Savant Vac centrifuge, Savant Instruments, Hicksville, NY, U.S.A.) and analysed by HPLC and the diode-array detection system.

HPLC and diode-array detection equipment

The HPLC system (LKB-Produkter AB, Bromma, Sweden) consisted of two LKB 2150 pumps, a LKB 2152 controller, a LKB 2152-400 high-pressure mixer and a LKB 2154 injector, together with the LKB 2140 rapid-scanning spectral detector interfaced with an IBM Personal Computer, equipped with a Canon A 1210 colorgraphics printer (Canon, NY, U.S.A.). The column (LKB 2134-630 Spherisorb ODS-2, particle size 3 μm, 100 × 4.6 mm I.D.) was eluted with a 20-min linear gradient of acetonitrile (0–50%) containing 0.1% trifluoroacetic acid (pH adjusted to 2.5 with sodium hydroxide). A flow-rate of 1.0 ml/min was maintained at a pressure of 140 bar.

RESULTS AND DISCUSSION

Fig. 1 illustrates the absorption spectra of tryptophan, phenylalanine and tyrosine standards obtained with the diode-array detector. As can be seen, and in

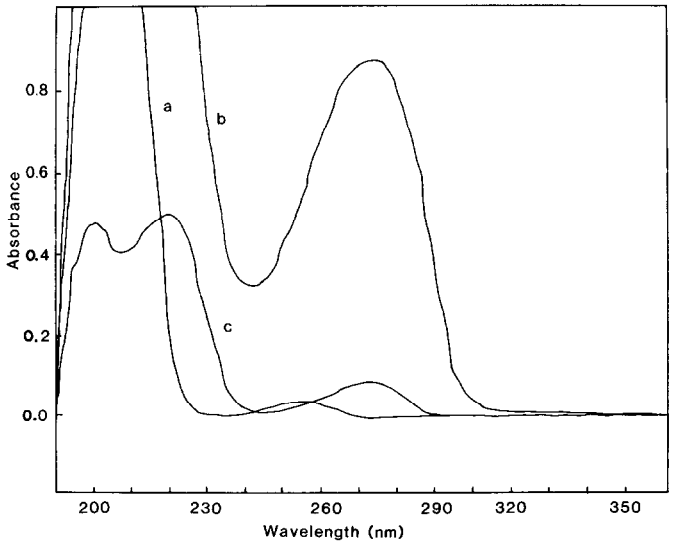

Fig. 1. Absorption spectra of standard amino acids. (a) phenylalanine; (b) tryptophan; (c) tyrosine. The detection limits for the amino acids were 1.0, 0.2 and 0.5 μg, respectively.

accordance with well-known facts, each of the analysed amino acids exhibits a characteristic absorption. Thus, since only the aromatic amino acids (except for cysteine) display appreciable natural absorption above 220 nm, peptides containing different aromatic residues should have different spectra. In this study, we used the ratio between absorption maxima and minima to identify the peptides with the respective aromatic amino acids. The absorption ratio of 255/270 nm was used for identification of tyrosine and tryptophan, whereas for phenylalanine the ratio of 255/265 nm was used (Table I). The absorbance value at 270 nm was not practicable for phenylalanine, because its absorption at this wavelength was too low to produce reliable ratio values. The absorbance ratios for synthetic peptides containing either tyrosine, tryptophan or phenylalanine, or some combination of these, were measured and used as reference values for the identification of the respective aromatic residues among the sample components (Table I).

TABLE I

ABSORPTION RATIOS OF HPLC-SEPARATED SYNTHETIC PEPTIDES AND AROMATIC AMINO ACIDS STANDARDS

Retention time (min)	*Substance*	*Absorption ratio* 255/270 nm*	*Absorption ratio* 255/265 nm*
6.32	Tyr	0.34 ± 0.01	
6.42	Tyr-Gly	0.34 ± 0.01	
6.42	Tyr-Gly-Gly	0.33 ± 0.01	
8.13	Phe	>1**	1.80 ± 0.13
10.25	Trp	0.61 ± 0.01	
12.21	Tyr-Gly-Gly-Phe	0.44 ± 0.01	
14.01	Gly-Gly-Phe-Leu	>1**	1.62 ± 0.10
14.25	Gly-Phe-Leu	>1**	1.62 ± 0.03
15.19	Tyr-Gly-Gly-Phe-Leu	0.42 ± 0.03	

* Values are given as means ± S.D. calculated from five different determinations.

** Due to low absorbance values for phenylalanine at 270 nm, its ratio 255/270 nm is not reliable, and tabulated values are only approximate.

Fig. 2 shows the HPLC separation of digestion products of (Leu)enkephalin with the ACE and enkephalinase-like activity in the presence of Captopril. UV spectra were recorded at two different wavelengths, 255 and 280 nm. The presence of Captopril (known to inhibit ACE) suggests that the conversion of (Leu)enkephalin was caused mainly by the enkephalinase activity. The chromatogram shown in Fig. 2 indicates that the reaction mixture was resolved into at least four distinct peaks. Peaks 1 and 3 are eluted with the enkephalin fragment Tyr-Gly (or Tyr-Gly-Gly) and (Leu)enkephalin itself, respectively, whereas peak 2 is eluted with the Gly-Phe-Leu fragment. The identity of these peaks with the above enkephalin fragments is confirmed by their absorption properties. Thus, peaks 1 and 3, containing tyrosine, show absorbance at both wavelengths, whereas peak 2, containing phenylalanine, shows absorbance only at 255 nm. The release of these fragments from (Leu)enkephalin by the hydrolytic activity of CSF has previously been observed by Hazato *et al.*[4]. In order to compare the difference in spectra between the three different peaks contain-

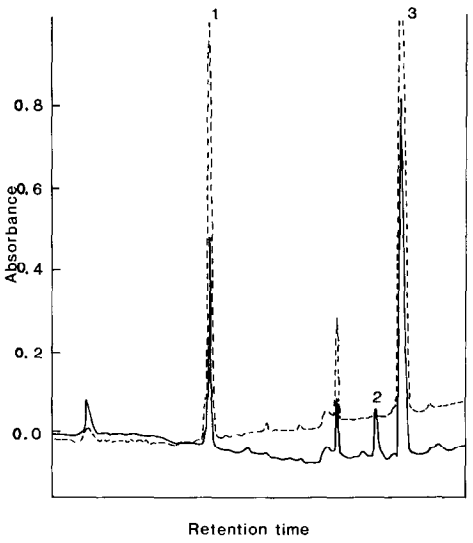

Fig. 2. HPLC separation of a digestion mixture after treatment of (Leu)enkephalin with CSF enzymes in the presence of Captopril. UV spectra were recorded at 255 nm (solid line) and 280 nm (dashed line).

ing phenylalanine, tyrosine and a combination of these residues, the spectrum was taken at the maximum of all three peaks (Fig. 3). The absorption ratios 255/270 nm and 255/265 nm, determined for each peak, were used to identify the respective aromatic amino acid or their combination (Table IIa). To verify the purity of the various peaks, the spectra of all peaks were taken on the upslope, maximum, and downslope. As is evident from the results presented in Figs. 4–6, the spectra are all identical,

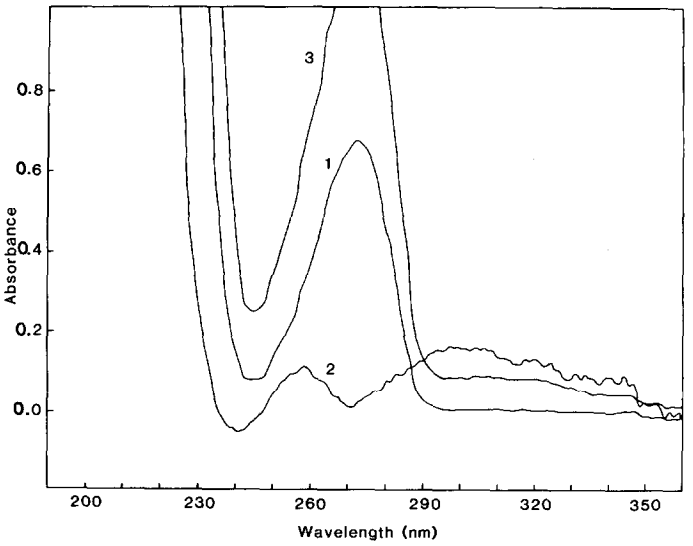

Fig. 3. Absorption spectra of peaks 1, 2 and 3 in Fig. 2.

TABLE II

ABSORPTION RATIOS OF HPLC-SEPARATED SAMPLE COMPONENTS

Retention time (min)	*Peak no.*	*Identified substance*	*Absorption ratio 255/270 nm*	*Absorption ratio 255/265 nm*
(a) Analysis of (Leu)enkephalin digest (see Fig. 2)				
6.49	1	Tyr-Gly	0.33	
14.13	2	Gly-Phe-Leu		1.40
15.19	3	Tyr-Gly-Gly-Phe-Leu	0.42	
(b) Analysis of DSIP and its Tyr^1 analogue (see Fig. 7)				
7.49	b	Tyr^1-DSIP(Tyr-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu)	0.33	
9.41	a	DSIP(Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu)	0.59	
(c) Analysis of dipeptides (see Fig. 9)				
2.54	1	Phe-Tyr	0.46	
3.07	1	Gly-Trp	0.58	

indicating a high purity and a constant amino acid composition of the three peaks. The unmarked peak that eluted ahead of peak 2 showed a spectrum which is different from any of those of the phenylalanine- and tyrosine-containing fragments. Furthermore, as the retention time of this peak coincided with that of Captopril, it is certainly not an enkephalin fragment but rather the inhibitor.

In an additional degradation experiment, the equipment was used to monitor the conversion of dynorphin A to (Leu)enkephalin-Arg^6 and Dyn A (7–17). The HPLC and diode-array detection system easily distinguished the tyrosine- from the tryptophan-containing fragment (not shown). The calculated ratios between absorption minima at 255 nm and absorption maxima at 270 nm were 0.43 and 0.58 for the N-terminal and C-terminal fragments, respectively (*cf.*, Table I).

Fig. 7 illustrates a separation between DSIP and its Tyr^1 analogue. The differences between the absorbance at the two different wavelengths is clearly shown. To identify the two peptides, the spectra were taken at the maximum for the two DSIP peptides; the different spectra are shown in Fig. 8. As can be seen, the substitution of the N-terminal tryptophan by a tyrosine residue is connected with a dramatic change in the spectrum of the peptide. The absorption ratios given in Table IIb provide a clear basis for discrimination between the two peptides.

In Fig. 9 a chromatogram of a mixture of two unresolved dipeptides is shown. The first part of the peak contains phenylalanyl-tyrosine and the second part glycyl-tryptophan. The absorption spectra taken from the lower frontal part of the peak

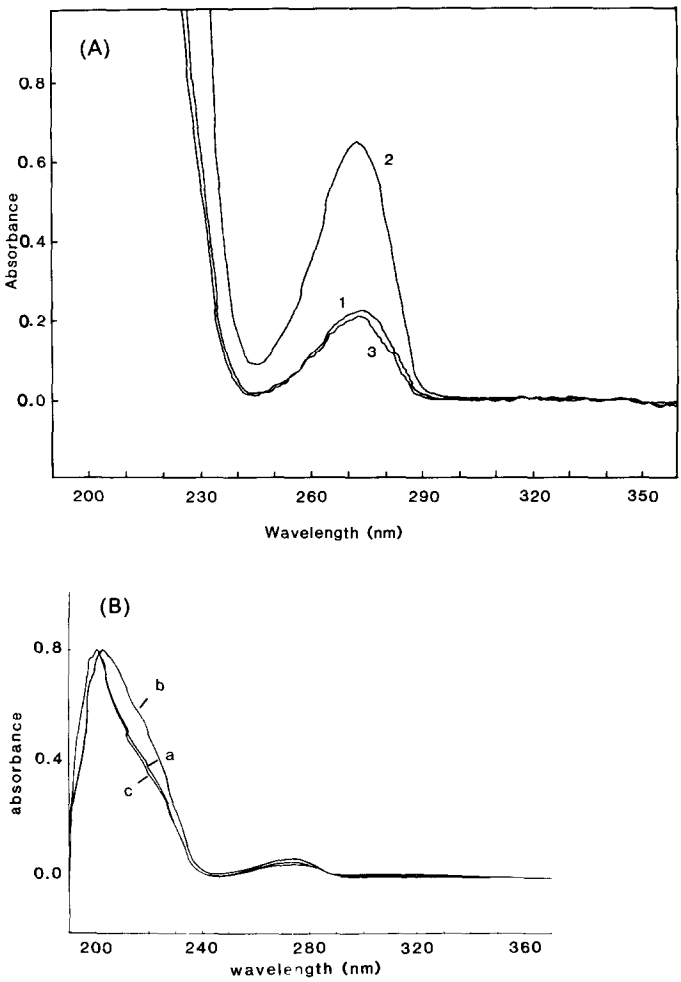

Fig. 4. Absorption spectra of peak 1 in Fig. 2, containing tyrosine. (A) Non-normalized spectra: 1, upslope; 2, maximum; 3, downslope. (B) Normalized spectra (scaled to 80% of full scale deflection): a, upslope; b, maximum; c, downslope.

(point 1) and at the maximum of the peak (point 2) were significantly different (not shown). The absorption measurements gave the ratios of 0.46 and 0.58, which is consistent with peptides containing tyrosine and phenylalanine in combination (0.44) and tryptophan (0.61), respectively (see Tables I and IIc).

In the experiments described here, we have run comparatively large amounts of peptides or peptide fragments and have not fully utilized the sensitivity of the detection system. It is known from previous studies that the diode-array detection technique allows analysis of peptides in concentrations below 1 nmol[1,9]. However, the aim of this study was to investigate the usefulness of the technique in degradation

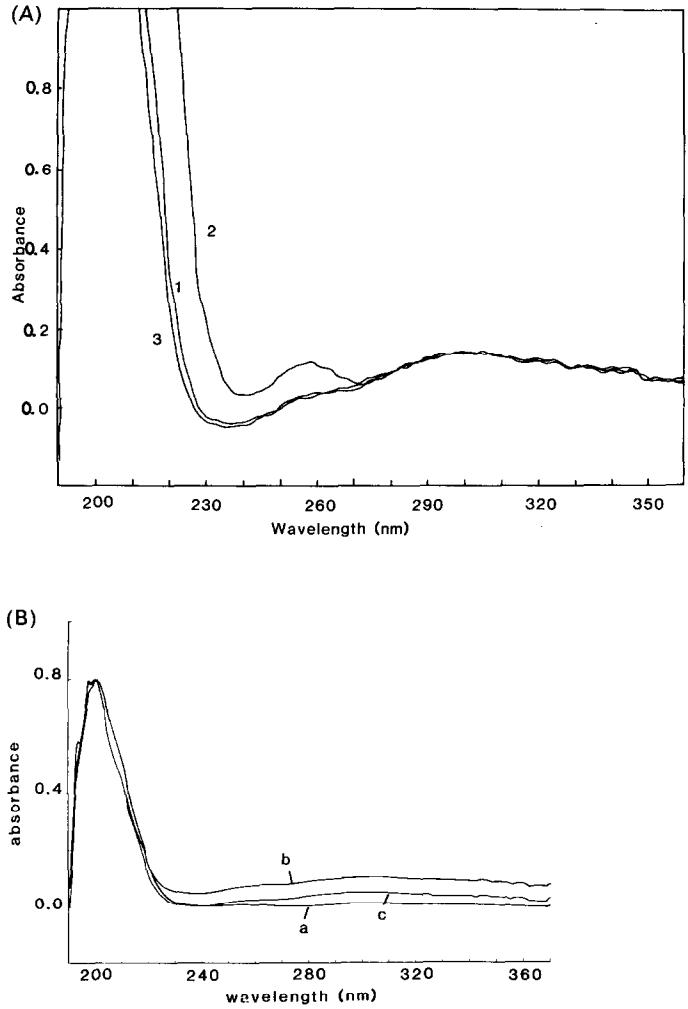

Fig. 5. Absorption spectra of peak 2 in Fig. 2, containing phenylalanine. (A) Non-normalized spectra: 1, upslope; 2, maximum; 3, downslope. (B) Normalized spectra (scaled to 80% of full scale deflection): a, upslope; b, maximum; c, downslope.

studies for discrimination of peptide fragments differing in their content of aromatic amino acid residues. The use of absorption ratios highly facilitated discrimination between spectra from peptides containing phenylalanine, tryptophan or tyrosine, or a combination of these residues (Table I). It should be noted, however, that the ratios may be affected by solvent and pH. For instance, the absorption maximum of phenylalanine has been shown to shift with pH alteration. It is therefore important to calibrate the HPLC diode-array detection system with amino acid and peptide standards for each buffer system to be used. From the spectra shown in Fig. 3 it appears

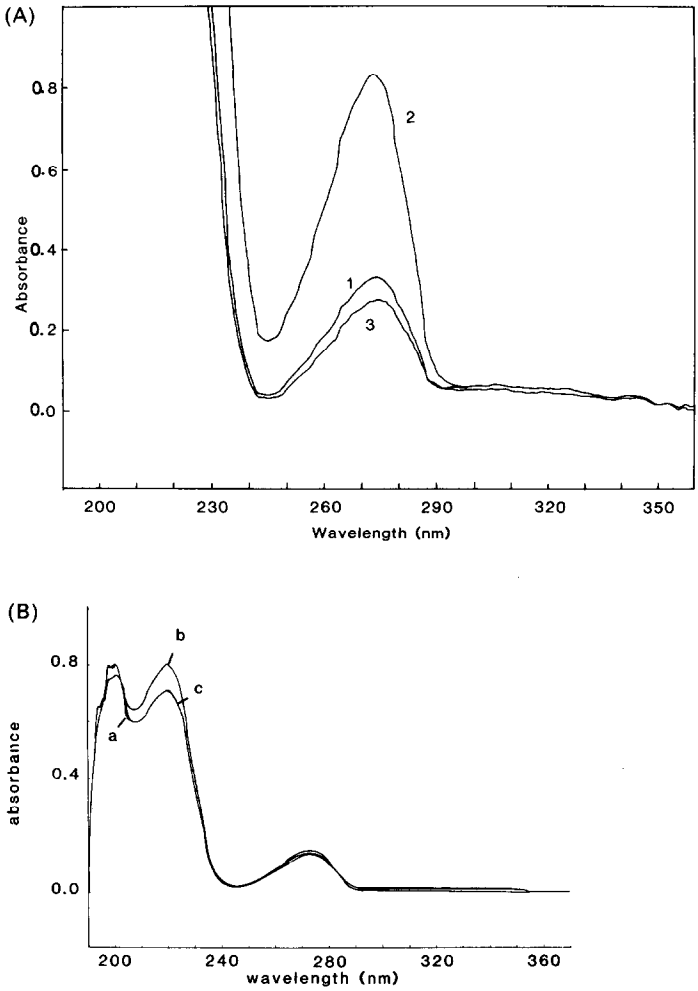

Fig. 6. Absorption spectra of peak 3 in Fig. 2. (A) Non-normalized spectra: 1, upslope; 2, maximum; 3, downslope. (B) Normalized spectra (scaled to 80% of full scale deflection): a, upslope; b, maximum; c, downslope.

that some peptides also exhibit absorption at wavelengths between 280 and 350 nm. This absorption was not observed in the runs of individual aromatic amino acids (Fig. 1). It is difficult to find a simple explanation for this but it may be due partly to solvent interaction since the peptides eluted from the HPLC column at a higher buffer concentration than did the amino acids. The additional absorption may also result from contribution of non-peptide material present in the enzyme preparation.

In conclusion, the results presented here indicate the usefulness of HPLC combined with diode-array detection in studies of neuropeptides. The introduction of diode-array detection provides important additional qualitative information for con-

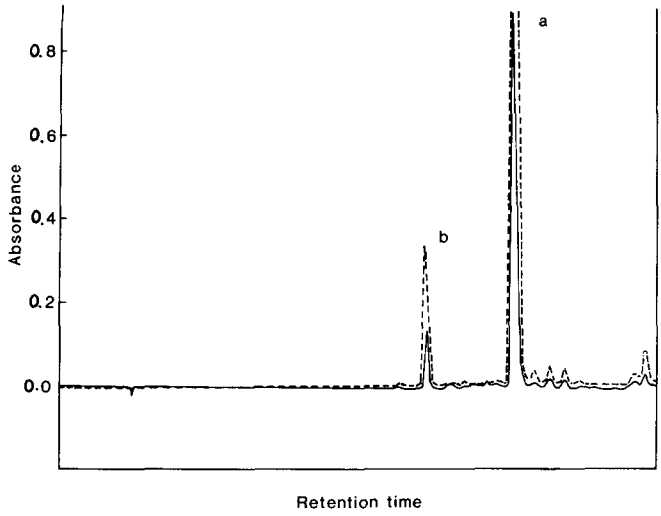

Fig. 7. HPLC of a sample containing equal amounts of (a) DSIP and (b) its Tyr[1] analogue recorded at 255 nm (solid) and 280 nm (dashed line).

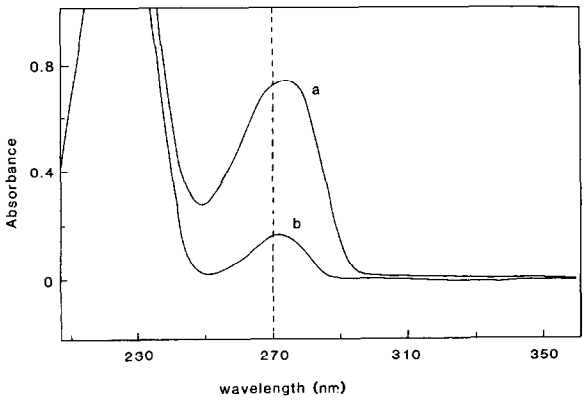

Fig. 8. Absorption spectra captured at peak maximum of (a) DSIP and (b) Tyr[1]-DSIP in Fig. 7.

firming the identity of peptide fragments as well as their parent compounds or analogues in various samples. The procedure proved to be highly efficient and reproducible and should be applicable to studies of many other peptide systems.

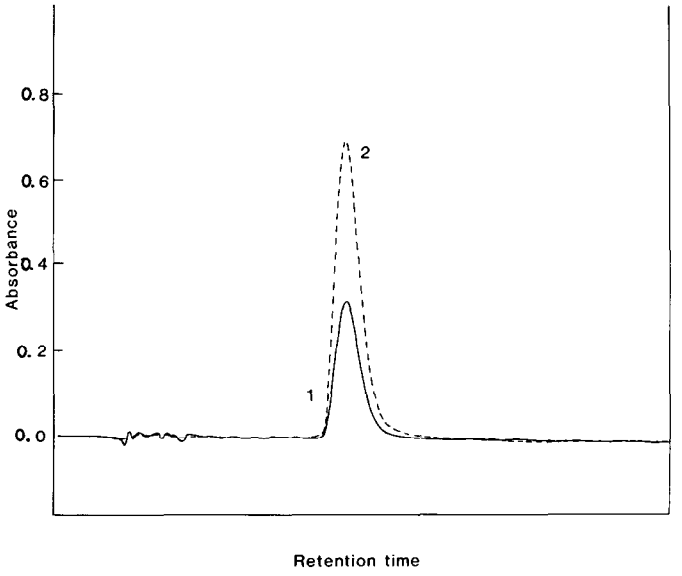

Fig. 9. HPLC of a mixture of two unresolved dipeptides: phenylalanyl-tyrosine and glycyl-tryptophan, recorded ast 255 nm (solid) and 280 nm (dashed line).

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