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ANALYSIS OF FLUORINE AND IODINE DERIVATIVES OF TYROSINE

T. J. CARNE*, R. E. HUBER, P. DAVITT and L. A. EDWARDS
Division of Biochemistry, Department of Chemistry, University of Calgary, Calgary, Alberta T2N 1N4 (Canada)

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SUMMARY

Separation of tyrosine, fluorotyrosine, monoiodotyrosine and diiodotyrosine was achieved by reversed-phase high-performance liquid chromatography (HPLC) using a gradient of acetonitrile with water and using trifluoroacetic acid for ion pairing. No derivatization of the amino acids, prior to separation, was needed. The spectral properties of Tyr and its fluorine and iodine derivatives and the dependence of their absorbance maxima on pH, made it possible to analyze and differentiate between these derivatives in the free amino acid form or in peptides. This analysis was accomplished by adjusting the post column HPLC eluate from two identical runs to different pH values and then comparing the spectra of the peaks from these two runs with a diode array detector. Hydrolysis in 6 *M* hydrochloric acid was totally destructive to mono- and diiodotyrosine. However, base hydrolysis in 13.5 *M* sodium hydroxide for 30 min at 121°C in an autoclave caused no destruction and allowed excellent recovery of all of the Tyr derivatives. This is the first report of simple methods for the detection and analysis of these amino acids and of a hydrolytic method which protects against their loss. A method of storage was also proposed.

INTRODUCTION

Fluorine and iodine derivatives of Tyr have recently gained importance in biochemistry and in molecular biology. Fluorotyrosine can replace Tyr in some proteins and the ^{19}F NMR spectra as well as the effects of the substitution on the activity of proteins can be studied[1–3]. Modification of Tyr by iodination is also used to determine the role of Tyr in proteins[4–6] and iodination of proteins using radioactive iodide is a very common method of labelling proteins[7]. In addition, the naturally occurring iodinated Tyr (the precursors of thyroxine) have been the object of several investigations[8].

The application of high-performance liquid chromatographic (HPLC) methods has made it possible to rapidly separate iodinated tyrosines and thyronines[9,10] and peptides containing iodinated Tyr[11]. Fluorotyrosine has also been separated from tyrosine by HPLC[12] but the method required that fluorotyrosine first be derivatized before separation. Methods for rapid identification of these substituted

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Tyr have, however, not kept pace with the separation methods. In studies done on iodine derivatives of free Tyr and thyronines, the identification can, of course, be accomplished by controls in which the retention times are predetermined but this is not practical as a means of identifying derivatives of Tyr which are present in peptides. It is possible to identify radioactively labelled iodinated Tyr in peptides separated by HPLC by the use of scintillation flow counters[6] and another method, using the catalytic properties of trace amounts of iodine on the reaction between cerium and arsenic has been used to detect iodinated compounds in HPLC eluates[13]. However, these two methods, although sensitive, are incapable of distinguishing between the mono- and diiodinated forms of Tyr and do not detect peptides containing uniodinated Tyr or fluorotyrosine.

Two other problems which are encountered when working with Tyr derivatives are those of hydrolysis and storage. Acid hydrolysis (6 *M* hydrochloric acid) of peptides and proteins will totally deiodinate any iodinated Tyr present in a sample[14]. To avoid this deiodination, hydrolysis of proteins containing iodinated Tyr has been carried out using proteases[8] which preserve the iodinated Tyr. The method, however, results in only partial hydrolysis of the protein and is time consuming. It has also been observed (unpublished observations) that iodinated Tyr becomes deiodinated over a period of weeks when stored in dilute acetic acid, a common solvent for storing tryptic peptides.

This paper presents simple, rapid methods for the identification of iodine and fluorine derivatives of tyrosine (alone or in peptides) in HPLC eluates. The methods are based on the use of either a diode array detector or simple UV spectra in conjunction with the absorbance properties of the Tyr derivatives at different pH values. No modification of these amino acids is necessary by the methods presented. We also present a method for the rapid, complete, non-destructive hydrolysis of proteins or peptides containing fluorine or iodine derivatives of Tyr. In addition, the studies presented here will show the best method of storing iodine derivatives of Tyr so that the derivatives are not deiodinated.

MATERIALS AND METHODS

Chemicals

Tyr and its iodine and fluorine derivatives, three Tyr containing peptides (Gly-Leu-Tyr, Tyr-Ala and Tyr-Glu), lactoperoxidase, and myoglobin were obtained from Sigma. Acetonitrile, HPLC grade, was obtained from Baker. Sequanal grade trifluoroacetic acid (TFA) was obtained from Pierce. Other reagents were from Fisher Scientific or similar sources. All reagents used were the purest grades available.

Iodination of peptides and myoglobin

For the Tyr containing peptides, the reaction mixture contained 2–3 mg/ml of peptide, 0.4 m*M* sodium iodide, and 25 μg/ml lactoperoxidase in 0.05 *M* phosphate buffer at pH 7.0. To initiate the iodination reaction, 5 μl/ml of a 0.06% solution of hydrogen peroxide was added. After 2 min, another identical aliquot of peroxide was added. At 4 min, the reaction was quenched by the addition of azide to 0.2%. In the case of myoglobin the reaction conditions were similar, the only differences being that the peroxide solution was 0.03% and aliquots were added every 4 min for 30 min.

Separation and detection

The separations of the substituted Tyr and of peptides containing these residues were carried out on a Hewlett-Packard 1084B high-performance liquid chromatograph equipped with a Whatman ODS reversed-phase column (5.0 μm, 4.6 × 250 mm). Elution was achieved with an acetonitrile gradient in water. All solvents contained TFA. Detection of the solutes in the eluate was performed by a Hewlett-Packard HP79875A variable-wavelength detector at either 220 nm or at 280 nm and by a Hewlett-Packard 1040A diode array detector which was used to obtain spectra of individual peaks and isoabsorbance plots of the elution profile. In some cases, a 0.2 *M* borate buffer at pH 9.5 or a 0.2 *M* carbonate buffer at pH 10.5 was added to the HPLC eluate with a Varian PCR1 pump and a mixing cell, prior to passing the eluate through the diode array detector. These additions resulted in final pH values of the eluents of 9 and 10, respectively.

Hydrolysis for amino acid analysis

Three different acids were tested for their effectiveness in hydrolyzing peptides containing substituted Tyr without degrading these residues. Samples were dissolved in 4 *M* methanesulphonic acid, 3 *M* toluenesulphonic acid, or 6 *M* hydrochloric acid with or without 0.2% phenol or cresol and sealed, under a vacuum, in glass tubes. The tubes were placed in an oven at 110°C for 24 h. Subsequent to hydrolysis, the tubes were opened and the contents were dried under a vacuum. The amino acids were then redissolved in water with 0.2% TFA buffer for HPLC analysis.

Base hydrolysis was carried out by two different methods[15,16]. In the first method, the samples were dissolved in 150 μl of 4 *M* sodium hydroxide and sealed in screw cap PTFE vials. They were then placed into an oven at 110°C for 18 h. In the second method, the samples were dissolved in 150 μl of 13.5 *M* sodium hydroxide in polypropylene tubes before being placed in an autoclave at 121°C for 30 min. After hydrolysis by either method, the samples were neutralized with acetic acid and diluted to 1 ml with water. Base hydrolysis in glass was not possible due to the etching of the glass[17].

Trypsin digestion of myoglobin

Iodinated myoglobin was dialyzed against 0.2 *M* ammonium bicarbonate (pH 8.5) and trypsin was added to the myoglobin solution at a ratio of 1:50 (w/w). The solution was kept at 37°C for 4 h at which time an identical aliquot of trypsin was added and the digestion was continued for an additional 20 h.

RESULTS

Separation and detection

Tyr and its fluorine and iodine derivatives were successfully separated on a reversed-phase Whatman ODS column by HPLC using a gradient of acetonitrile and water (Fig. 1A). The eluate from the HPLC was directed through a diode array detector where the spectrum, from 210 nm to 400 nm in increments of 2 nm, was memorized every 15 s and stored on a flexible disk. This data was then expressed in a variety of forms including isoabsorbance plots and as spectra of individual peaks. Fig. 1 shows these isoabsorbance plots of Tyr and its fluorine and iodine derivatives

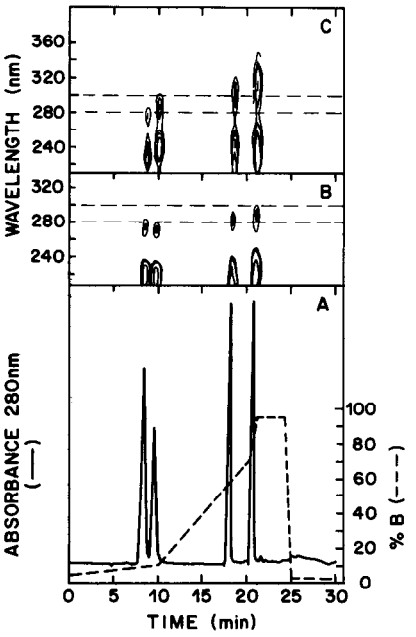

Fig. 1. Separation of Tyr derivatives by HPLC. (A) Elution profile (280 nm) of Tyr derivatives. The order of elution is Tyr, fluorotyrosine, monoiodotyrosine, and diiodotyrosine. Solvent A was water with 0.2% TFA. Solvent B was acetonitrile with 0.1% TFA. The gradient was as shown. Separation was on a Whatman ODS reversed-phase column as described in the Materials and methods section. (B) Isoabsorbance plot of the Tyr derivatives obtained on the diode array detector at pH 2.0 for the separation described above. (C) Isoabsorbance plot of the Tyr derivatives obtained on the diode array detector after the eluent was adjusted to pH 9 for the separation described above.

at pH 2 (B) and of a sample in which the eluate was adjusted to pH 9 prior to passing it through the diode array detector (C). Spectra of each of the four amino acid peaks are shown in Fig. 2. The three sets of spectra, A, B and C (Fig. 2) were obtained when the pH values of the eluates were 2, 9 and 10, respectively. It can be seen from both the isoabsorbance plots (Fig. 1) and the spectra (Fig. 2) that there was a shift in the absorbance maxima of the spectra for the three Tyr derivatives when the pH was adjusted from 2 to 9. This is a result of the differences in the pK_a values of the hydroxyl groups of these residues and the dependence of their absorbances on the dissociation of the hydroxyl group. Since the pK_a of Tyr itself is above 9, a shift in its spectra cannot be observed until the pH is raised to 10 (Fig. 2C). Table I lists the pK_a values and the absorbance maxima for Tyr and its derivatives. The pK_a of fluorotyrosine was determined for this study. The absorbance maxima listed in the table were taken from the spectra obtained from the diode array detector.

A slightly different acetonitrile to water gradient was used to separate the iodinated forms of the Tyr containing peptides. Fig. 3 shows an elution profile and the acetonitrile gradient for the iodinated peptide, Gly-Leu-Tyr, and isoabsorbance plots obtained at pH 2 and pH 9. The spectra for each peak at the two pH values are shown in Fig. 4. From the isoabsorbance plots (Fig. 3) and the spectra (Fig. 4), it is obvious that the first peak represents the non-iodinated peptide while the second

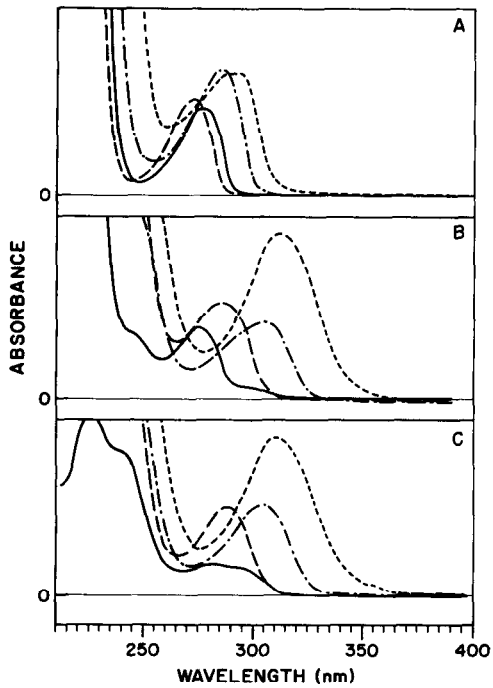

Fig. 2. Spectra of Tyr derivatives obtained from the diode array detector. Separation on HPLC was described in Fig. 1. (A) Spectra at pH 2.0: (———) Tyr (8.31 min), (– – –) fluorotyrosine (9.57 min), (– – – – –) monoiodotyrosine (17.99 min), (·····) diiodotyrosine (20.58 min). (B) Spectra at pH 9.0 achieved by post-column addition of 0.2 *M* borate buffer at pH 9.5 prior to detection. (C) Spectra at pH 10.0 achieved by post-column addition of 0.2 *M* carbonate buffer at pH 10.5 prior to detection.

and third peaks are the mono- and diiodinated forms, respectively. The broad monoiodotyrosine peak indicates that the pK_a of the hydroxyl group is higher in this peptide than in the free amino acid. Similar results were obtained for the peptides, Tyr-Ala and Tyr-Glu. The elution times obtained are in agreement with the fact that iodination of peptides causes them to become more hydrophobic and thus elute from a reversed-phase column later than the less iodinated forms of the same peptides[19].

TABLE I

THE pK_a VALUES OF TYR AND ITS DERIVATIVES WITH THEIR ABSORBANCE MAXIMA AT pH VALUES ABOVE AND BELOW THE pK_a

The starred values were taken from the literature[18] and the remainder of the values were obtained in this study.

Amino acid	*pK_a*	*Absorbance maxima (nm)*	
		Below pK_a	*Above pK_a*
Tyrosine	10.1*	274	295
Fluorotyrosine	8.3	272	290
Monoiodotyrosine	8.7*	285	305
Diiodotyrosine	6.5*	290	315

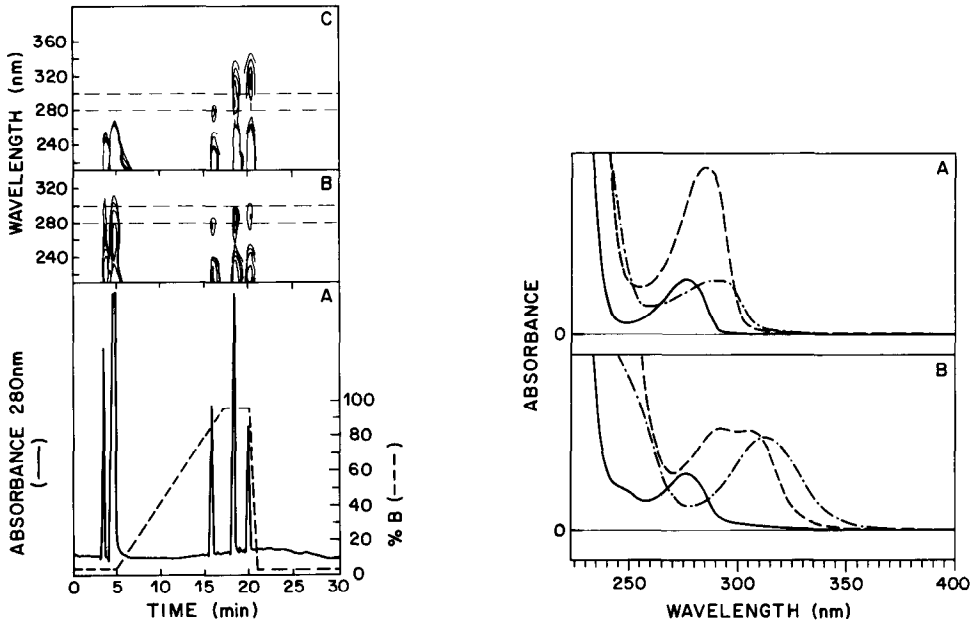

Fig. 3. Elution profile (280 nm) of iodinated Gly-Leu-Tyr on HPLC. Separation was on a Whatman ODS reversed-phase column as described in the Materials and methods section. The same solvents were used as in Fig. 1. (A) Elution profile (280 nm) of the separated peptides. The order of elution was the non-iodinated peptide (15.60 min) followed by monoiodinated (18.06) and diiodinated (19.65) peptides. The large amount of material at the solvent front was excess reagent. (B) Isoabsorbance plot of the peptides obtained at pH 9 as described in Fig. 2B. (C) Isoabsorbance plot of the peptides obtained at pH 10 as described in Fig. 2C.

Fig. 4. Spectra of iodinated Gly-Leu-Tyr derivatives obtained from the diode array detector following separations described in Fig. 3. (A) Spectra at pH 2.0: (———) desiodo Gly-Leu-Tyr (15.60 min), (– – –) monoiodo Gly-Leu-Tyr (18.06 min), (– – – – –) diiodo Gly-Leu-Tyr (19.65 min). (B) Spectra at pH 9.0 (as above).

Since Trp is the only amino acid which might interfere with the detection and identification of Tyr (Phe elutes very differently and has a very weak absorbance) it was added to a solution containing the Tyr derivatives and eluted according to the gradient in Fig. 1. The Trp eluted with monoiodotyrosine as a single, uniform peak. However, the presence of monoiodotyrosine in this peak could be demonstrated by spectra of the peak at pH 2 and pH 9 as shown in Fig. 5. This demonstrated that the peak, at pH 9, contained a component which has an absorbance maximum at 279 nm and another component with a maximum at 305 nm. Thus, if Trp and monoiodotyrosine were in one peptide or in one peak containing several peptides, their presence could be detected in this manner. In the case of the free amino acids, Trp and monoiodotyrosine were found to be separated by using an isocratic elution with 20% acetonitrile.

Storage

Past experience in the laboratories of the authors indicated that when peptides containing iodinated Tyr are stored in acidic solution, deiodination occurs slowly

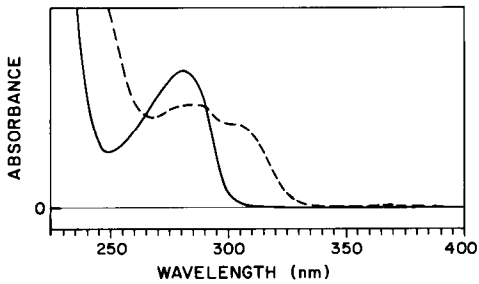

Fig. 5. Spectra of monoiodotyrosine in the presence of tryptophan at pH 2.0 (———) and pH 9.0 (– – –) obtained from the diode array detector. The presence of Trp is indicated by the absorbance maximum at 279 nm at pH 9 while the presence of monoiodotyrosine is indicated by a maximum at 305 nm. The concentration of Trp was one third that of monoiodotyrosine.

(within a manner of weeks). Thus it was decided to study the stability of such peptides when stored at neutral pH. Samples of each of the three iodinated peptides were run on the HPLC and then the remaining solution was separated into three parts. One part was frozen, a second stored at 4°C and the last was left at room temperature. Each sample was rerun on the HPLC after 20 days. In every case, the amounts of the des-, mono-, and diiodinated forms of the three peptides were identical to the controls. Thus the iodinated Tyr appear to be stable at neutral pH and are best stored at such a pH rather than at low pH.

Hydrolysis

It has been previously reported that hydrolysis in 6 *M* hydrochloric acid of peptides containing monoiodotyrosine or diiodotyrosine results in complete deiodination of these residues[14]. Therefore, different methods of acid hydrolysis were tested on Tyr, fluorotyrosine, monoiodotyrosine and diiodotyrosine. Methanesulphonic acid (4 *M*), *p*-toluenesulphonic acid (3 *M*), and 6 *M* hydrochloric acid were used in the presence and the absence of the protective agents cresol or phenol. The results verified that the iodine derivatives of Tyr were completely deiodinated by hydrolysis in 6 *M* hydrochloric acid even in the presence of 0.2% cresol or phenol. Fluorotyrosine, however, was stable and could be quantitated following acid hydrolysis. The only acid hydrolysis method which showed any significant protection of the iodine derivatives was 4 *M* methanesulphonic acid with 0.2% phenol. Thus this method was tested on the iodinated peptides. Elution profiles of these hydrolysates showed the presence of some mono- and diiodotyrosines but they also showed the presence of considerable amounts of Tyr itself, indicating that some destruction of iodinated Tyr had occurred. Also, the presence of UV absorbing materials in the methanesulphonic acid [including phenol and 3-(2 aminoethyl)indole added by the supplier] made quantitation difficult. Acid hydrolysis does not, therefore, appear to be a useful method for obtaining free Tyr and its derivatives in a form suitable for analysis.

Several variations of base hydrolysis were then tested in order to find a method which resulted in complete hydrolysis of the peptides with a minimal amount of deiodination. The common method of base hydrolysis, that of exposing peptides to 4 *M* sodium hydroxide at 110°C for 18 h, resulted in complete hydrolysis (no peaks were

TABLE II

RECOVERIES OF TYROSINE AND ITS DERIVATIVES AFTER BASE HYDROLYSIS IN 13.5 *M* SODIUM HYDROXIDE FOR 30 min IN AN AUTOCLAVE

The recoveries are reported as averages of the percent of total recovery plus or minus the standard error of the mean for several (4–9) experiments.

Amino acid	*Recovery (%)*
Tyrosine	73 ± 6
Fluorotyrosine	77 ± 4
Monoiodotyrosine	68 ± 4
Diiodotyrosine	64 ± 6

observed where the original peptide eluted). However, in addition to the expected amino acid peaks, new, unidentified peaks appeared which probably represented rearrangements of the amino acids. The method of choice was found to be hydrolysis in 13.5 *M* sodium hydroxide for 30 min in an autoclave at 121°C. This procedure resulted in excellent hydrolysis, there was no loss of iodine or fluorine from the derivatives, and none of the unidentified peaks were observed. The recoveries of the amino acids after hydrolysis by this method are listed in Table II. The values represent averages of several hydrolysis experiments using both iodinated Gly-Leu-Tyr peptide derivatives and the free amino acids themselves. The recovery of fluorotyrosine compared well to the recovery of Tyr while the recoveries of mono- and diiodotyrosine were slightly lower but still within experimental error. Most of the losses were due to the handling of small samples as was shown by a control experiment in which Tyr was carried through the entire procedure excluding only the autoclave treatment. The observed recoveries are adequate for a typical amino acid analysis since the important result is usually molar ratios of amino acids rather than absolute values. If absolute values are important the inclusion of an internal standard is suggested.

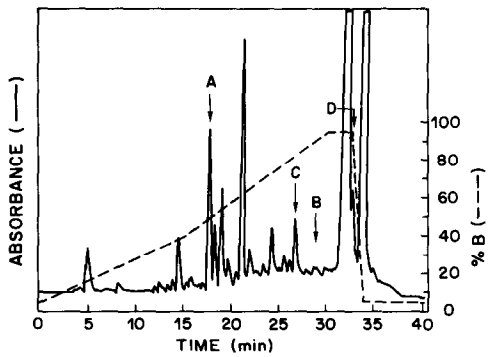

Fig. 6. Elution profile (280 nm) of tryptic peptides of myoglobin separated by HPLC on a Vydac 218 TP reversed-phase column. The solvents were the same as those described in Fig. 1. The gradient was as indicated. Peaks labelled A, B, C and D correspond to examples of peptides containing des-, mono- and diiodotyrosine and a diiodotyrosine-containing peptide which was incompletely separated from one containing Trp.

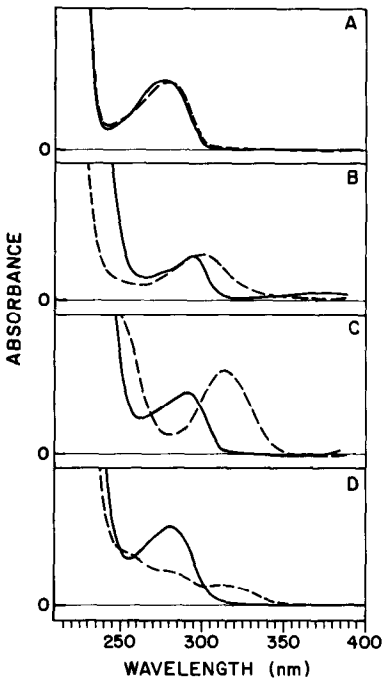

Fig. 7. Spectra from the diode array detector of the tryptic peptides from myoglobin which were identified in Fig. 6. Spectra were obtained at pH 2 (———) and pH 9 (– – –). These peptides contain (A) a Tyr (uniodinated), (B) a monoiodotyrosine, (C) a diiodotyrosine, and (D) a peptide containing diiodotyrosine which was incompletely separated from one containing Trp.

Myoglobin: a test case

In order to test these techniques, myoglobin was iodinated and digested with trypsin. This digest was then chromatographed on the HPLC system on a VYDAC reversed-phase column and the eluate was passed through the diode array detector. Fig. 6 shows the elution profile at 280 nm of the tryptic peptides of myoglobin. Spectra of the four peaks which are labelled in Fig. 6 were obtained when the eluate was pH 2 and when the eluate was adjusted to pH 9 prior to passing through the diode array detector. These are shown in Fig. 7. Fig. 7A shows a typical Tyr peak. In Fig. 7B, the spectrum is seen to shift to slightly higher wavelengths as the pH is increased, which is typical of monoiodotyrosine. A greater shift in the spectrum is seen in Fig. 7C which is typical of diiodotyrosine. Inspection of Fig. 6 shows that the peak, designated D, was not completely separated from a very large peak. It can be seen from the spectra shown in Fig. 7D that, at pH 9 there is a maximum at 315 nm, typical of diiodotyrosine, but there is still a peak at 280 nm which must be the Trp of the very large adjacent peak. (Myoglobin contains two Trp residues in one tryptic peptide which would constitute a very large peak at 280 nm).

DISCUSSION

The increased usage of Tyr derivatives and problems such as identification and

deiodination of these derivatives under acid conditions[14], has led to the need for improved methods for the identification, storage, and hydrolysis of these compounds. Presented here are rapid and simple methods, requiring no additional derivatization, for the identification and for non-destructive hydrolysis of Tyr derivatives. A method for storage of these compounds is also presented.

Tyrosine, its fluorine and iodine derivatives, and peptides containing the iodine derivatives were shown to be separated by reversed-phase chromatography on HPLC. One of the most common methods for determining the identity of chromatographed peptides containing iodinated Tyr is through the use of radioactive iodine. For the detection of naturally occuring iodinated Tyr or thyronines, a method exists which makes use of the catalytic properties of iodine in the reaction between cerium and arsenic. Although these methods are very sensitive, they cannot be used to determine the degree of iodination of Tyr in peptides. A single peptide can appear three times on a chromatogram; in its non-iodinated, mono-, and diiodo forms[18]. By using the methods described in this paper, these residues, as well as fluorotyrosine, were readily identified by comparing their absorbance maxima as determined at pH 2 and at pH 9. These maxima are properties of the dissociation of the hydroxyl groups and thus dependent on the pK_a of the group. The pK_a values of Tyr and its iodine derivatives are available in the literature[17]. The pK_a of fluorotyrosine was determined for this study and is, to our knowledge, the first reported pK_a for this amino acid.

There are several ways to display the data obtained by a diode array detector for a given analysis of peptides or amino acids. A three dimensional plot of elution time *vs.* wavelength *vs.* absorbance (not shown) gives a qualitative overview of the data but precise, quantitative data is difficult to extract from such a plot. The isoabsorbance plots, seen in Figs. 1 and 3, display data about the entire chromatogram and immediately indicate the identity of Tyr derivatives. This study indicated that the most precise method for determining absorbance maxima and thus the identity of a derivatized Tyr and the presence of a combination of Tyr derivatives and/or Trp is by comparing the spectra of individual peaks at different pH values. The shapes of the spectra at pH values above and below the pK_a values of the substituted Tyr are very sensitive to the particular residues which are present. In the absence of a diode array detector, the data obtained in this study, as listed in Table I and displayed in the spectra of Figs. 2 and 4, can be used to choose single wavelengths at which absorbances can be measured and compared at different pH values and thus be used to identify the residue which is present. Alternatively, if the HPLC system in use is equipped to do so, the flow can be stopped at a given peak and a spectrum may be taken in order for the Tyr derivative in that peak to be identified. If such an instrument is not available, however, the eluate could be collected and spectra of the fractions containing Tyr or its derivatives could be obtained on a spectrophotometer.

It was determined that the iodine derivatives of Tyr are stable when stored at neutral pH and are stable to base hydrolysis, when carried out under the conditions described, even when oxygen is not excluded from the reaction mixture. Fluorotyrosine is stable to either base or acid hydrolysis. Since the Tyr derivatives are stable to base hydrolysis, it is possible to perform a reliable amino acid analysis of a purified peptide which has been shown to contain one or more of these Tyr derivatives.

The feasibility of the methods discussed above, for identifying Tyr derivatives, was tested by iodinating myoglobin, digesting it with trypsin and separating the pep-

tides by reversed-phase chromatography. The success of the method was demonstrated by obtaining the spectra in Fig. 7 which identify samples of peptides containing des-, mono- and diiodotyrosine as well as demonstrating the presence of a peak containing both diiodotyrosine and Trp.

In summary, simple methods for the separation, identification, storage, and hydrolysis of Tyr and its iodine and fluorine derivatives were described which should be useful in research in the many fields which now use these amino acids as probes.

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