Dityrosine: Preparation, Isolation, and Analysis

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Dityrosine is an unusual amino acid that is distinguished by the intense 400-nm-range fluorescence of its singly ionized form, measurable upon excitation within either 315 nm (alkaline solutions) or 284 nm (acidic solutions) absorption bands (1). The generation of dityrosine in proteins is a normal physiological process in specialized cases and a result of exposure to environmental agents in others. Dityrosine cross-links are naturally occurring in proteins from the elastic ligaments of insects (2), the ascospore cell walls of Saccharomyces cerevisiae (3), the cell walls of Candida albicans (4), the cuticles of Haemonchus contortus (5) and Ascaris suum (6), and the fertilization envelope of the sea urchin egg Stronglyocentrotus purpuratus (7). Vertebrate animal proteins known to contain dityrosine include elastin (8), collagen (9), and a storage form of thyroglobulin (10).

Dityrosine may be a useful marker for assessing oxidative damage to proteins (11, 12). Agents that promote the in vitro formation of dityrosine include ultraviolet irradiation (13-17), OH and N3 radicals (18, 19), NO2 (20), peroxynitrite-ONO0 (21), and lipid hydroperoxides (22). Exposure to H2O2 results in the conversion of globin tyrosyl residues to dityrosine in the case of metmyoglobin (23) or of oxyhemoglobin (11).

Enzyme-catalyzed phenolic coupling may find applications in the chemical modification and in vitro cross-linking of proteins. Horseradish peroxidase catalyzes the in vitro production of dityrosine in several proteins—including chymotrypsin and insulin (24). Highly efficient cross-linking of proteins (e.g., calmodulin, troponin C, the 20-kDa smooth muscle myosin light chain) that are not recognized by most of the common peroxidases is attainable with a peroxidase from Arthromyces ramosus (25).

Thus, studies involving the posttranslational modification of proteins, oxidative stress, or in vitro protein cross-linking may require chemical analyses for dityrosine. Reverse-phase HPLC (4, 5, 12, 26, 27), anion-exchange HPLC (28), and ion-exchange on Aminex AB resin (29) have been employed for this purpose. In addition, precolumn derivatization of protein hydrolysates

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with dabsyl chloride followed by reverse-phase HPLC gives a complete amino acid analysis that includes di-
tyrosine (30). However, the existence of overlapping peaks and/or the necessity of processing relatively large quantities of hydrolyzed protein make the application of these techniques to samples containing trace amounts of dityrosine difficult. In any case, the interpretation of the results requires a standard: authentic dityrosine.

Our interest in both uv-irradiation-induced and en-
zyme-catalyzed dityrosine formation in calmodulin (15–17, 25) led us to consider effective ways of dealing with dityrosine in protein hydrolysates. This article describes several chromatographic procedures which we have developed that have multiple applications in the preparation of dityrosine, in the isolation or enrichment of dityrosine from protein digests and hydrolys-
ates, and in dityrosine analysis per se. It includes improvements in the preparative-scale enzymatic synthesis of dityrosine.

**MATERIALS AND METHODS**

Reagents and Proteins

Dabsyl chloride, amino acid standards, L-tyrosine, Mops, 1 boric acid, β-mercaptoethanol, horseradish per-
oxidase, horseradish superoxide dismutase, and A. ra-
mosus peroxidase were purchased from Sigma Chemi-
Cal Co. 2,2'-Dihydroxy-biphenyl was supplied by Fluka Chemika. Acetonitrile, 2-propanol, and trifluoroacetic acid (HPLC grades) were purchased from Baker Chem-
cal Co. Highly purified water was obtained from a Milli-Q system (Millipore). All other reagents (formic acid, acetic acid, KCl, sodium pyrophosphate, sodium hydroxide, and H2O2) were of the highest purity avail-
able. Bovine brain calmodulin was prepared according to established procedure (31). A polymerized fraction of calmodulin, free of monomer, was prepared using the Arthromyces peroxidase-catalyzed reaction of Malencik and Anderson (25).

Affinity Chromatography on Immobilized Phenyl Boronate

We packed a column with Matrex Gel PBA-60 (Ami-
con Corp.) (1 × 5 cm) which had been equilibrated in a buffer consisting of 0.01 M sodium pyrophosphate, 0.50 M KCl, pH 8.2 to 8.3 (25°C). Amino acid mixtures or protein hydrolysates (containing in our examples 2.1 to 2200 nmol of dityrosine) are dissolved in 0.5 ml of the equilibration buffer just before application to the column. The pH of this mixture is checked and adjusted to 8.1–8.3 if necessary. The column is run (25°C) ini-
tially in the equilibration buffer, with a change to 0.10 m sodium borate (in 0.01 M sodium pyrophosphate, pH 8.2 to 8.3, 0.5 M KCl) or to 0.01 M sodium pyrophosphate alone made as soon as the elution of tyrosine is com-
plete (just after the collection of fraction 11). The flow rate was 12 ml/h and the fraction sizes averaged 0.75–
0.76 ml.

**Isocratic Reverse-Phase HPLC**

An isocratic reverse-phase HPLC system (LKB 2150) also was used to analyze dityrosine in conjunction with both absorbance (Isco V4) and fluorometric (HP 1046) detection systems. The reverse-phase column, ODS II Spherisorb from LC-Resources, has 11% carbon loading, 5-μm particle size, 100 Å pore size, and overall dimensions of 4.6 × 250 mm. The solvent used in this case consists of 92% water, 8% acetonitrile, and 0.1% trifluoroacetic acid (25°C). By employing an excitation wavelength of 280 nm, the excited-state ionization characteristic of dityrosine allows fluorometric moni-
toring of acidic solutions (see later section on fluo-
rescence monitoring).

**Amino Acid Analysis**

Hydrolysis of calmodulin samples was performed in sealed (N2) glass ampoules containing 1.5 ml 6 N HCl plus 0.25% phenol. Side by side hydrolysates were per-
formed on two samples: one consisting of 13.5 mg of cross-linked calmodulin and the other (a standard and control) consisting of 13.5 mg of native calmodulin plus added dityrosine (695 nmol). Hydrolysis was carried out for 48 h in a 120°C oven. Hydrochloric acid was removed from the hydrolyzed samples by lyophili-
ization.

Total amino acid analyses (for up to 22 amino acids including dityrosine, phosphotyrosine, phosphoserine, and phosphothreonine) were carried out using reverse-
phase HPLC according to our modification (30) of the method of Knecht and Chang (32). This procedure en-
tails precolumn derivatization of protein hydrolysates

4 In this case, we hydrolyzed a relatively large quantity of cross-
linked calmodulin in order to have a supply of protein-derived dityro-
sine for study. Ordinarily, we hydrolyze samples containing 1–10 μg of calmodulin (30).
TABLE 1
Rapid Gradient Program for the Reverse-Phase HPLC
Analysis of Dabsylated Dityrosine

| Solvent A | 20 mM KH₂PO₄, pH 5.5, with 4% dimethylformamide, 1.5% 2-propanol, and 13.5% acetonitrile |
| Solvent B | 90% acetonitrile + 10% 2-propanol |
| Temperature | 40°C |
| Flow rate | 1 ml/min |

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>% A</th>
<th>% B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>70</td>
<td>30</td>
</tr>
<tr>
<td>15</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>15.1</td>
<td>30</td>
<td>70</td>
</tr>
<tr>
<td>17.1</td>
<td>30</td>
<td>70</td>
</tr>
<tr>
<td>18.0</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

at 70% B (67% acetonitrile). In carrying out the fast gradient program, we employ a Phenomenex Ultrasphere 20 HPLC column (4.6 × 250 mm), which is characterized by 20% carbon loading, a particle size of 5 μm, and a pore size of 100 Å. As in our previous procedure (30), we monitored the absorbance at 460 nm (Isco V4 detector) and determined peak areas using the Scientific Software data acquisition system (Version 6.2).

Fluorometric Monitoring

Table 2 summarizes the three types of fluorometric monitoring applied to the detection of dityrosine. Measurements in alkaline solutions, employing excitation and emission wavelengths of 320 and 400 nm, give maximum sensitivity. Dityrosine has a ground-state phenolic pKₐ near 7 (1). In pH 9.7 buffers, more than 99% of dityrosine is present as the fluorescent singly ionized form. Dityrosine also undergoes ionization in the excited state (14), with an apparent pKₐ < 3 (33). Thus, excitation at the 284-nm absorption band of un-ionized dityrosine makes it possible to detect dityrosine in acidic solutions. The emission spectrum of dityrosine is independent of pH since the emitting species is always the singly ionized form (14). Excitation and emission wavelengths of 301 and 377 nm correspond, respectively, to the isosbestic and isoemissive points found in the absorption and fluorescence emission spectra of dityrosine in the presence of varying concentrations of boric acid–sodium borate. The fluorescence intensities obtained with this combination of wavelengths are essentially independent of the latter concentrations. Fluorescence measurements using excitation and emission wavelengths of 280 and 300 nm, respectively, identify possible tyrosine-containing fractions.

The monitoring of the time courses and of chromatography on DEAE–cellulose, BioGel P-2, and Matrex Gel PBA-60 was performed manually with the Perkin–Elmer LS-50 luminescence spectrophotometer. Standard curves of fluorescence versus the concentration of dityrosine for the various combinations of buffer and wavelength are linear up to concentrations of ~4 μM (10-mm-path cuvettes). When the samples were monitored without dilution, the concentration of dityrosine was always less than 4 μM. More concentrated solutions were diluted in 0.10 M NaHCO₃–Na₂CO₃ (pH 9.7), giving final dityrosine concentrations of 10 μM or less.

Absorption Spectra

Baseline-corrected absorption spectra were determined with the Perkin–Elmer Lambda 3B UV/Vis Absorption Spectrophotometer.

Enzyme-Catalyzed Preparation of Dityrosine

This stepwise procedure is based on information given under Results.

Step 1. Add 452 mg of L-tyrosine to 400 ml of distilled water in a 1-liter Erlenmeyer flask. Using a hot

TABLE 2
Fluorometric Monitoring of Dityrosine

<table>
<thead>
<tr>
<th>Excitation λ (nm)</th>
<th>Emission λ (nm)</th>
<th>Applications and conditions</th>
<th>Relative sensitivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>280</td>
<td>400</td>
<td>Low pH, reverse-phase HPLC</td>
<td>10–17</td>
</tr>
<tr>
<td>320</td>
<td>400</td>
<td>Diluted samples in 0.10 M NaHCO₃–Na₂CO₃, pH 9.7</td>
<td>100</td>
</tr>
<tr>
<td>301</td>
<td>377–378</td>
<td>Varying boric acid–sodium borate concentrations; phenyl boronate chromatography</td>
<td>42</td>
</tr>
<tr>
<td>280</td>
<td>300–305</td>
<td>Detection of tyrosine</td>
<td>—</td>
</tr>
</tbody>
</table>

* These values may vary with the instrumentation used.
plates, bring this suspension to boiling, continuing until all of the tyrosine has dissolved. Cool the solution to 30–40°C and add 100 ml 0.5 M boric acid–sodium borate, pH 9.1. Bring the total volume to 500 ml, thus obtaining a 5 mM solution of tyrosine in 0.1 M borate buffer, pH 9.1. (Solutions may contain up to ~8 mM tyrosine before the solubility limit is exceeded.) Place the flask in a constant temperature bath maintained at 38–40°C.

Step 2. Dissolve 10.0 mg of peroxidase from either A. ramosus or horseradish in 10 ml of H2O. Add the enzyme to the 5 mM tyrosine solution and mix gently. Immediately add 1.42 ml of 3% H2O2 (0.5 mol H2O2/mol tyrosine) and briefly swirl the solution. (The same results are obtained if the H2O2 is added stepwise rather than all at once. Prolonged agitation results in enzyme inactivation.) Remove 2-μl samples of this mixture at various times after the addition of H2O2, diluting each aliquot with 2.0 ml of 0.1 M sodium bicarbonate that has been adjusted to pH 9.7. Determine the fluorescence intensities of the diluted samples, employing excitation and emission wavelengths of 320 and 400 nm, respectively. When the fluorescence intensity no longer changes (Fig. 1), add 175 μl of 0.2 M mercaptoethanol to the reaction mixture. Divide this golden brown solution between two 600-ml lyophilizer flasks, freeze over liquid nitrogen, and lyophilize to dryness. (The latter facilitates removal of β-mercaptoethanol and residual H2O2, if present.)

Step 3. Dissolve the lyophilized material in 250 ml of distilled H2O and adjust the pH to 8.8. Apply this solution to a column of Whatman DEAE-cellulose (2.75 × 19.5 cm) that has been equilibrated in 0.02 M NaHCO3, pH 8.8, and elute with 0.2 M boric acid–sodium borate, pH 8.8. Under these conditions, most of the pigmented by-products adsorb while tyrosine and dityrosine appear in the pale yellow breakthrough fractions. Pool and lyophilize the dityrosine-containing samples. This step facilitates steps 4 and 5 through removal of material that would adsorb strongly to BioGel P-2.

In preparation for step 4, suspend the lyophilized powder in 20 ml of cold distilled H2O, mix by stirring, transfer the suspension to a 30-ml Corex centrifuge tube, and spin at 5000 rpm for 10 min in a Sorvall RC-2B centrifuge. Decant and save the supernatant extract obtained. Use an additional 15 ml of cold H2O to rinse the interiors of the lyophilizer flasks and to resuspend the precipitate remaining in the centrifuge tube. Combine the supernatant solution obtained upon centrifugation of this mixture with the prior extract. (The residue may be saved for the future extraction of additional small quantities of dityrosine.) Adjust the pH of the combined extracts to ~7 by adding formic acid (88%). Allow this solution to stand overnight at 0°C and remove any precipitate formed by centrifugation.

Step 4. Apply the supernatant solution to a column of BioGel P-2 (200–400 mesh, Bio-Rad) (4 × 34.5 cm) that is equilibrated and run in 0.10 M NH4HCO3 (4°C), using a flow rate of 40 ml/h. Collect fractions containing 5.5 ml. Absorption measurements, made on undiluted samples, monitor the fractionation of 370-nm-absorbing contaminants. Fluorescence measurements on diluted samples demonstrate the elution of dityrosine as a sharp peak centered near fraction 60. Measurements at wavelengths corresponding to the excitation (280 nm) and emission (300 nm) maxima of tyrosine, performed on 20-μl aliquots diluted in 2.0 ml 25 mM Mops, pH 7.3, show a peak centered near fraction 84. We pooled and lyophilized dityrosine-containing fractions 57 through 72 (Fig. 2A).

Step 5. Dissolve the lyophilized dityrosine obtained in step 4 in 20 ml of 0.10 M HCOOH. Add concentrated HCOOH (88%) to the solution until the pH is less than 3. (Note that the pH must be less than 3. BioGel P-2 chromatography of dityrosine carried out at pH 4 gave the same results obtained at pH 7 (34).) After removing any precipitate that appears, apply this pH-adjusted solution to the column of BioGel P-2 (4 × 34.5 cm), which now is equilibrated and run (40 ml/h) in 0.10 M HCOOH (4°C). Dityrosine elutes as a peak centered near fractions 98–99 (Fig. 2B). We pooled fractions 95–104 and 105–113, to be lyophilized and stored in a desiccator at −20°C. One hundred twenty milligrams of dityrosine, representing 26.5% of the maximum theoretical yield, is thus recovered. Note that the storage of dityrosine in the solid form is important. Solutions of dityrosine deteriorate over a period of days. In preparation for future use, the BioGel P-2 column is washed with a solution containing 25% acetone–75% water and then reequilibrated.

Chemical Characterization of the Purified Dityrosine

Fast atom bombardment mass spectrometry (performed in the Oregon State University Environmental Health Sciences Mass Spectrometry Laboratory) shows that both pools contain a predominant component of mass 361, a minor component of mass 383 (which is probably a sodium adduct), and no component exhibiting the mass of trityrosine. Reverse-phase HPLC analysis (Fig. 3) according to our isocratic procedure (Materials and Methods) shows that each pool contains only one 400-nm-emitting component and only one 280-nm-absorbing component. Elemental analysis (conducted by Desert Analytics, Tucson, AZ) of a dried sample representing fractions 95–104 gave 55.28 ± 0.02% C, 5.36 ± 0.04% H, 7.27 ± 0.03% N, and 23.99 ± 0.35% O. The theoretical values are 59.99% C, 6.12%H, 7.73% N, and 26.48% O. This analysis is consistent with a composition of ~91.9% dityrosine and ~8.1% of components containing elements other than C, H, N, and O.
**RESULTS**

**Enzyme-Catalyzed Synthesis of Dityrosine**

The step-by-step method for the preparation and purification of dityrosine detailed under Materials and Methods is based on new information that we reserved for presentation in Results and on the first step in the procedure of Amado et al. (29). Figure 1 contains time courses showing the enzyme-catalyzed conversion of tyrosine to dityrosine obtained with both horseradish and A. ramosus peroxidases (Scheme 1).

The extent of reaction obtained with the two enzymes (41% conversion based on the assumption that all of the fluorescence is due to dityrosine and that the colored by-products do not interfere with the measurements) is similar. Although the reaction rates differ, even the slower reaction catalyzed by the horseradish enzyme is complete within a period of time (25-30 min) that is much shorter than the 24-h incubation period previously employed (29). Other modifications to the original reaction conditions include reduction of the boric acid–sodium borate concentration from 0.2 M (pH 9.5) to 0.1 M (pH 9.1), done in order to reduce the quantity of residue that must be extracted later, and reduction of the H$_2$O$_2$ from 1 mol H$_2$O$_2$/mol tyrosine to the ideal stoichiometry of 0.5 mol H$_2$O$_2$/mol tyrosine. Significantly higher fluorescence yields result from the latter change. The inset to Fig. 1 shows that the apparent reaction yield has a broad pH optimum, centered closer to pH 9 than to pH 9.5. Since superoxide dismutase stimulates the A. ramosus peroxidase-catalyzed production of dityrosine in calmodulin (25), we also monitored reaction mixtures containing 20 µg/ml horseradish peroxidase plus 20 µg/ml horseradish superoxide dismutase. No change in rate or yield resulted.

**pH-Dependent Chromatography of Dityrosine on BioGel P-2**

We encountered several disadvantages while carrying out the purification of dityrosine according to Amado et al. (29). The recommended chromatography on cellulose–phosphate CP-11 spreads dityrosine over many fractions containing 0.5 M NaCl while chromat...
PREPARATION, ISOLATION, AND ANALYSIS OF DITYROSINE

**Scheme 1**

\[
\begin{align*}
2 \text{ Tyrosines} & \quad + \quad \text{H}_2\text{O}_2 \quad \rightarrow \quad \text{Dityrosine} & \quad + \quad 2\text{H}_2\text{O} \\
\end{align*}
\]

chromatography on Dowex 50-X8 leads to losses of 35% of the applied dityrosine. Our chance discovery of the pH-dependent chromatography of dityrosine on BioGel P-2 is a key feature of the purification procedure outlined under Materials and Methods. Application of a crude mixture that had been subjected to DEAE-cellulose chromatography in order to remove pigments (step 3) to a BioGel P-2 column equilibrated in 0.10 M NH₄HCO₃ leads to the expected order of elution of dityrosine and tyrosine (Fig. 2A). However, both components partially overlap a broad band of residual pigment detected in the absorption measurements.

Working on the premise that dityrosine undergoes pH-dependent associations with reaction by-products, we carried out a second fractionation in which the dityrosine-containing fractions from step 4 (lyophilized and redissolved) are applied to a column of BioGel P-2 equilibrated in 0.10 M HCOOH. Figure 2B shows the unanticipated results thus obtained. At low pH, dityrosine exhibits marked retention by the gel matrix, with the elution volume (∼545 ml at the peak) considerably exceeding the total volume of the column (∼430 ml). This phenomenon facilitates the further separation of small molecules (salts, etc.) and of 300-nm-emitting impurities from dityrosine. The yellow contaminants eluted represent about 13% of the total 370-nm absorbance applied, with the rest being adsorbed by BioGel P-2. (The change in pH alone does not appreciably affect the absorbance at 370 nm.)

To determine whether the pH-dependent chromatography on BioGel P-2 could be useful for the isolation of dityrosine from protein digests and hydrolysates, we applied a 1.0-ml sample containing 2.1 μmol of dityrosine and 2.5 μmol each of 17 other amino acids (Ala, Arg, Asp, cystine, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Tyr, and Val) to a small column (1.5 × 47 cm) of BioGel P-2 equilibrated and run in 0.10 M NH₄HCO₃. We pooled, concentrated, and analyzed the 400-nm-emitting fractions obtained (tubes 40–43). Total amino acid analyses performed on dabsylated samples (30) showed that this pool includes trace amounts of just three other amino acids: Tyr, Pro, and Val.

**Spectral Characterization of the Purified Dityrosine**

Dityrosine is highly soluble in both acidic and basic solutions. Concentrated solutions, containing 50 mg/ml or more dityrosine, are pale yellow. Absorption spectra, recorded over a range of pH values that bracket the ground-state pKₐ of dityrosine, exhibit well-defined isosbestic points (Fig. 4). On the basis of the elemental analyses, we calculate minimum extinction coefficients for dityrosine of 8380 ± 40 cm⁻¹ M⁻¹ at 315 nm (pH 9.9 in 0.10 M NaHCO₃–Na₂CO₃) and of 5680 ± 30 cm⁻¹ M⁻¹ at 283 nm (in 0.05 M KH₂PO₄ adjusted to pH 4). The concentrations of the dityrosine standards used throughout this work are based on dry weight, with consideration of the results of the elemental analyses, or on absorbance measurements obtained with alkaline (pH 9.9) solutions. The preceding extinction coefficients compare to values that we have determined for the absorption maxima of 2,2'-dihydroxy-biphenyl [8300 cm⁻¹ M⁻¹ at 307 nm (pH 9.9) and 5500 cm⁻¹ M⁻¹ at 278 nm (pH 4)]. The inset to Fig. 5 shows the fluorescence emission spectrum of the purified dityrosine. Note that the emission spectrum is independent of pH since the emitting species is always the singly ionized dityrosine chromophore, produced by either ground-state (high pH) or excited-state (low pH) ionization.

**Affinity Chromatography of Dityrosine on Immobilized Phenyl Boronate**

We tried chromatography on Matrex Gel PBA-60, an immobilized phenyl boronate designed for the fractionation of small diol-containing ligands (35), as
FIG. 2. Fractionation of dityrosine on BioGel P-2. (A) Separation obtained when a partially purified sample containing ~126 mg of dityrosine, tyrosine, and unidentified components is applied to a column of BioGel P-2 (4 × 34.5 cm) that is equilibrated and run in 0.10 M NH₄HCO₃ (4°C). Fraction size was 5.5 ml. (B) Fractionation of the dityrosine pool recovered from A (tubes 57–72, lyophilized and redissolved) on a BioGel P-2 column (4 × 34.5 cm) that is equilibrated and run in 0.10 M HCOOH (4°C). The three types of monitoring are absorbance at 370 nm, performed on undiluted samples (dashed line); fluorescence intensity at 400 nm, performed on 1000-fold diluted samples (refer to Fig. 1 legend); and fluorescence intensity at 300 nm (excitation: 280 nm), performed on samples diluted 100-fold in 25 mM Mops, pH 7.3.

TABLE 3
Recovery of Dityrosine after Chromatography on Immobilized Phenyl Boronate and on BioGel P-2

<table>
<thead>
<tr>
<th>Column</th>
<th>Sample</th>
<th>Elution method</th>
<th>Amount of DiY recovereda</th>
</tr>
</thead>
<tbody>
<tr>
<td>BioGel P-2</td>
<td>2.2 μmol DiY, 25 μmol each of 17 amino acids</td>
<td>0.10 M NH₄HCO₃</td>
<td>2.2 μmol</td>
</tr>
<tr>
<td>BioGel P-2</td>
<td>0.83 μmol DiY, residual amino acids</td>
<td>0.10 M HCOOH</td>
<td>0.83 μmol</td>
</tr>
<tr>
<td>PBA-60</td>
<td>3.5 nmol DiY, 25 nmol Y</td>
<td>0.10 M boric acid–borate</td>
<td>3.5 nmol</td>
</tr>
<tr>
<td>PBA-60</td>
<td>2.5 nmol DiY, 25 nmol Y</td>
<td>0.20 M boric acid–borate</td>
<td>2.39 nmol</td>
</tr>
<tr>
<td>PBA-60</td>
<td>2.5 nmol DiY, 25 nmol Y</td>
<td>Low ionic strength</td>
<td>2.50 nmol</td>
</tr>
</tbody>
</table>

a Error ±5%.

an analytical tool and as a means of isolating dityrosine from protein digests and hydrolysates. Dityrosine undergoes an interaction with boric acid–sodium borate solutions that is consistent with the formation of a 1:1 diester complex. Maximum interaction occurs near pH 8, with an apparent dissociation constant for the complex of ~17 mM (34). The inset to Fig. 5 shows the effect of a 0.20 M boric acid–sodium borate buffer on the fluorescence emission spectrum of dityrosine, obtained with an excitation wavelength (301 nm) corresponding to the isosbestic point in the absorption spectra of dityrosine and the dityrosine–boric acid complex (34). Experiments with mixtures of tyrosine (50 nmol) and...
dityrosine (2 to 3.5 nmol)\textsuperscript{5} demonstrate an interaction of the latter with phenyl boronate that apparently involves both specific association with the boronate moiety and hydrophobic binding. Under the conditions used in Fig. 5 (0.01 M sodium pyrophosphate, 0.5 M KCl, pH 8.2, 25°C), tyrosine is eluted with the breakthrough fractions (peak near tube 10) while dityrosine is retarded, ultimately appearing as a broad band spanning more than 20 fractions. When present, tryptophan (detected fluorometrically) and all of the other normal amino acids [detected by total amino acid analysis of the dabsylated derivatives (30)] elute with tyrosine. We show two different procedures for the elution of dityrosine as a concentrated pool, centered near fraction 21, which is well separated from tyrosine. Both involve a change in the elution buffer, made just after the collection of fraction 11.

In one case, 0.1 to 0.2 M boric acid–sodium borate is added to the elution buffer and in the other case, KCl is omitted, leaving a solution containing only 0.01 M sodium pyrophosphate, pH 8.2 (Fig. 6). Essentially 100% recovery of the applied dityrosine is obtained with either approach (Table 3). In experiments that are not shown, we also have eluted dityrosine by adjustment of the pH, to values less than 7 or greater than 9. However, the reequilibration of Matrex Gel PBA-60 requires larger quantities of buffer than are needed with the other elution methods. Increases in the length of the column (from 1 × 5.1 cm to 1 × 12 cm) do not significantly improve the separations.

We prepared two hydrolysates—one originally containing 13.5 mg of native calmodulin plus 695 nmol (see footnote 5) added dityrosine (0.86 mol DiY/mol CaM) and the other containing 13.5 mg of calmodulin (see footnote 4) that had been reacted with H\textsubscript{2}O\textsubscript{2} in the presence of A. ramosus peroxidase (25). Phenyl boronate chromatography, with elution by boric acid–sodium borate, of samples representing 67.5 μg of the original protein yields 2.64 nmol of dityrosine from the control and 2.21 nmol of dityrosine from the oxidized sample. Since the recovery of dityrosine standards from PBA-60 is essentially 100% and the control initially contained 3.48 nmol dityrosine per 67.5 μg, approximately 24% of the dityrosine must have been lost during hydrolysis. By assuming that the loss is the same for both samples, we calculate that the oxidized calmodulin contains an average of 0.72 mol dityrosine/mol CaM.

\textsuperscript{5} Concentrations are based on information regarding composition and extinction coefficients given under Results.
protein. Matrex Gel PBA-60 chromatography (using the same column) of a sample representing 6.8 mg of oxidized calmodulin gives 242 nmol of dityrosine (0.78 mol dityrosine/mol protein).6

The results of isocratic reverse-phase HPLC performed on a 0.8-nmol sample of the dityrosine that we had recovered from PBA-60 chromatography of the oxidized calmodulin hydrolysate is shown in Fig. 3. A 75-pmol sample of the recovered dityrosine also is used in an illustration of the rapid gradient program for the reverse-phase HPLC analysis of dabsylated dityrosine (Fig. 6). Note that all of the other dabsylated amino acids, except for tyrosine, elute with the unreacted reagent as a single unresolved peak at 34–37% solvent B.

DISCUSSION

We have described procedures that are invaluable for the preparation, isolation, and analysis of dityrosine. We have obtained a minimum of 26.5% (120 mg dityrosine from 452 mg tyrosine) of the maximum theoretical yield of purified dityrosine from the enzyme-catalyzed oxidation of tyrosine. This yield is twofold larger than that achieved through the procedure reviewed by Amado et al. (29). The two methods of preparing dityrosine lead to products that are generally similar (±10%) in purity. However, HPLC analyses of dityrosine prepared according to the earlier approach demonstrate a second 400-nm-emitting species, eluting at higher concentrations of acetonitrile (Malencik and Anderson, unpublished results). This component, which may be trityrosine (5), is absent in our case.

The two-dimensional chromatography of dityrosine on BioGel P-2 (Fig. 2) is the single most important aspect of our preparation procedure. Recent purifications of dityrosine employ thin-layer chromatography (12) or reverse-phase HPLC (4, 5, 12, 26, 27). However, neither method is capable of handling preparative-scale quantities. We find that overloading of reverse-phase HPLC columns occurs with ~50 μg or less of dityrosine. In addition, HPLC requires defined samples that do not contain major quantities of extraneous material (36). Chromatography on BioGel P-2 may be useful for the isolation of dityrosine from biological fluids, cell wall digests, protein hydrolysates, etc. As the result

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6 Hydrolysates of native calmodulin contain no components eluting in the position of dityrosine.
of chromatography performed in the two dimensions, the dityrosine obtained may be nearly pure or at least refined enough for further characterization by HPLC.

Affinity chromatography on immobilized phenyl boronate (Matrex Gel PBA-60) also is a procedure for the separation of dityrosine from tyrosine and other amino acids that has both preparative and analytical applications. Phenyl boronate chromatography was developed originally for the fractionation and analysis of diols that are capable of forming reversible, covalent complexes with boric acid–monoborate ion (35, 37, 38). Phenyl boronate chromatography of a hydrolysate of cross-linked calmodulin, prepared in a reaction catalyzed by A. ramosus peroxidase (25), yields a range of dityrosine that is consistent with the mixed population of polymers present. Since mammalian calmodulin contains two tyrosyl residues, the maximum possible amount of dityrosine ranges from 0.5 mol per mole calmodulin for a purely dimeric population to 1 mol per mole for either infinite polymerization or intramolecular cross-linking (25). Immobilized phenyl boronate chromatography of dityrosine has an advantage over gel filtration in that separations and measurements are completed within a 2-h time period. However, the nonvolatile solutes used interfere with dabsylation whenever the dityrosine eluted is highly dilute. The availability of phenyl boronate HPLC media (Progel-TSK Boronate-5 PW) may facilitate a novel HPLC analysis for dityrosine.

For analytical purposes, we also devised an isocratic reverse-phase HPLC procedure—employing a solvent containing 92% H₂O, 8% acetonitrile, and 0.1% trifluoroacetic acid—that gives excellent separations of tyrosine and dityrosine (Fig. 3). The lower limit for the determination of dityrosine in this solvent is ~10–20 pmol. The simplicity of isocratic reverse-phase HPLC,
FIG. 6. Reverse-phase HPLC analysis of dabsylated dityrosine according to the rapid gradient program. Samples shown are dityrosine (dabsylated), 75 pmol recovered from hydrolysate of cross-linked calmodulin (—) and tyrosine (dabsylated), 200 pmol (-----). The inset shows the proportionality between integrated peak areas and varying amounts of a dabsylated dityrosine standard (10 to 75 pmol). Column: Phenomenex Ultracarb 20. Solvents and gradient program: Refer to Table 1. (Note that a 5-min delay needs to be added to the times shown on the x-axis). Monitoring: absorbance at 460 nm.

which requires only a single pump, is the major advantage that this technique has over existing HPLC procedures (4, 5, 12, 26, 27) for the determination of dityrosine.

Reverse-phase HPLC of dabsylated protein hydrolysates gives a complete amino acid analysis that includes dityrosine (30). A modification of that procedure, following the rapid gradient program outlined in Table 1, enhances the separation between dabsylated dityrosine and the other amino acid derivatives. The inset to Fig. 6 shows the peak areas determined for samples containing from 10 to 72 pmol of dabsylated dityrosine. In our previous report, we analyzed hydrolysates of uv-irradiated calmodulin containing 1.5 pmol of dabsylated dityrosine (30). Thus, reverse-phase HPLC analyses for small quantities of dityrosine are possible when fluorescence detection systems are not available.

REFERENCES

lower limit of ~2 pmol of dityrosine when the excitation wavelength is 320 nm.