Characterization of Proteases by Capillary Electrophoresis with Laser-Induced Fluorescence Detection

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Introduction
Proteases are enzymes which catalyze the hydrolysis of peptide bonds. Proteases are often present in complex biological mixtures such as fermentation broths, serum, or urine. In biotechnology, protease contamination may present a serious problem when desirable proteins are purified. Traditionally, analysis of protease activity involves chromogenic or fluorogenic substrates to assay enzyme activity. Most protease substrates are peptides linked to ortho- or para-nitroanilide through amide bonds. Protease digestion of the substrate results in the formation of ortho- or para-nitroaniline which absorbs light significantly more strongly than does the substrate. By designing a nitroanilide peptide with a specific amino acid sequence, a protease may be probed. However, at very low protein concentrations (i.e., \(10^{-12}\) M), the enzymatic activity is difficult, if not impossible, to determine with traditional spectrophotometric methods. This is because the conversion of substrate to product is insignificant compared to the existing background of the substrate.

In this Application Bulletin (and in a recent paper\(^2\)), we propose to use a fluorescently labeled peptide as a stable substrate for the analysis of low-level proteases. The enzyme hydrolysis is monitored by P/ACE\(^\text{TM}\) capillary electrophoresis with laser-induced fluorescence detection (CE-LIF). The fluorophore used to label the substrate is Cy3, a cyanine-type dye which has an absorption maximum close to the helium-neon laser (543 nm) or a solid-state, frequency-doubled diode laser (532 nm). The laser light sources can be easily connected to the P/ACE system via standard fiber optic couplers.

Protease assays using CE with UV absorbance detection have been described.\(^3,4\) However, LIF detection is orders of magnitude more sensitive than UV, and should be, therefore, well suited for low-level analyte detection.

Experimental

Materials

Cy3, a carboxyl activated cyanine dye, was purchased from Biological Detection System (Pittsburgh, PA). All buffer components, angiotensin I, angiotensin II, and trypsin were products of Sigma Chemical Co. (St. Louis, MO). Proteinase K, carboxypeptidase P and Y were purchased from Boeringer Mannheim (Indianapolis, IN).
CE-LIF
A P/ACE 2100 instrument equipped with laser-induced fluorescence (LIF) detection (Beckman Instruments, Inc., Fullerton, CA), was used for the CE runs. Post-run data analysis was performed with Gold™ (version 7.0) software. Capillary columns, 27 cm length (20 cm to detector window) × 20 μm i.d. (Polymicro Technologies, Phoenix, AZ) were assembled in a P/ACE LIF cartridge. This cartridge contains an ellipsoidal mirror to collect fluorescence. A 15-mW, frequency-doubled diode laser emitting at 532 nm was kindly provided by Scott Miller of Amoco Laser (Naperville, IL). A laser headcoupler to a standard SMA-906 fiber connector to the PACE system with LIF detector was a product of OZ optics (Ontario, Canada). The fluorescence signal was collected through a narrow-band filter of 590 nm ± 9 nm (Oriel, Stratford, CT) while the laser beam was rejected by a notch filter at 532 nm (Applied Physics, Torrance, CA). A 5-mW, “green” helium-neon laser (543 nm) was purchased from Particle Measurement Systems (Boulder, CO) and a notch filter at 543.5 nm was purchased from Barr Associates (Westford, MA).

Synthesis of Cy3-Angiotensins
Cy3, an activated carboxyl cyanine dye, was coupled to the N-terminal of the peptides. Angiotensin I was dissolved in 50 mM phosphate buffer (pH 7.5) at a final concentration of 1.0 mg/mL. 50 μL of the angiotensin I solution (equivalent to 46 nmol) was added to a vial of the activated Cy3 (80 nmol) at room temperature for 30 minutes. The resulting mixture was chromatographed on a C-18 reversed-phase column (4.1 mm × 25 cm, Beckman ODS Spherogel). Peaks containing peptide and Cy3 were collected from the liquid chromatograph (System Gold®). Purity was assessed by LC, CE, and UV/Visible spectrophotometry. Similarly, Cy3-angiotensin II and Cy3-aspartic acid were synthesized by the above procedures.

Protease Digestion
Cy3-angiotensin I (10⁻⁸ M, in 90 μL of 0.05 M Tris-HCl buffer, pH 8.0) was mixed with 10 μL protease K (0.1 U/mL) at room temperature. The reaction mixture was immediately monitored by CE-LIF. Samples were introduced by pressure injection for 20 s. Electrophoresis was performed in a 200 mM borate buffer, pH 10.2, with a field strength of 740 V/cm (20 kV/30 μA). The capillary was maintained at ambient temperature (23°C) in the P/ACE cartridge. Between runs, the capillary was sequentially rinsed under high pressure with 1.0 N sodium hydroxide and water (12 s each), followed by reconditioning with borate buffer for 60 s.

Trypsin and carboxypeptidase catalyzed hydrolysis of Cy3-angiotensin I and II were performed similarly.

Results

Digestion of Cy3-Angiotensin I and II with Trypsin and Carboxypeptidase P
The purity of the labeled substrates, Cy3-angiotensin I (asp-arg-val-tyr-ile-his-pro-phe-his-leu) and Cy3-angiotensin II (asp-arg-val-tyr-ile-his-pro-phe), was first confirmed by CE. As can be seen in Figure 1A, the migration times of Cy3-angiotensin I (peak 1) and Cy3-angiotensin II (peak 3) are 2.1 and 2.25 minutes, respectively. Cy3 diacid (migration time 4.2 min) is present in each sample as a reference. Addition of trypsin to the above mixture results in the formation of a single Cy3-peptide, as shown in Figure 1B. The specificity of trypsin digestion suggests that this peptide is Cy3-asp-arg (peak 9). Addition of carboxypeptidase P to the trypsin digest mixture led to the formation of Cy3-asp (peak 10, Figure 1C).

![Figure 1](image-url)

Figure 1. A: CE-LIF electropherogram of Cy3-angiotensin I and II, 10 nM each; Cy3 diacid, 1.0 nM. Laser light source: frequency-doubled semiconductor laser, emitting at 532 nm (Amoco Laser). B: Tryptic digest of Cy3-angiotensin I and II, 10 nM each; Cy3 diacid, 1.0 nM. C: Carboxypeptidase digest of the reaction mixture in Figure 1B. Peak i.d.: see Table 1; run voltage: 20 kV.
Digestion of Cy3-Angiotensin I with Proteinase K
Proteinase K is a powerful protease capable of degrading native proteins, even at high pH or in the presence of SDS or urea. The enzyme acts as an endo- as well as an exopeptidase. The time profile of enzymatic digestion of the substrate (Cy3-angiotensin I) by proteinase K can be monitored in Figure 2. Immediately after the addition of proteinase K (approximately 0.1 min), about 30% Cy3-angiotensin I is hydrolyzed to Cy3-angiotensin II (data not shown). Eight minutes after the addition of proteinase K, nearly all Cy3-angiotensin I is converted to Cy3-angiotensin II (peak 3, migration time 2.25 min) along with the formation of a minor product, Cy3-asp-arg-val-tyr, appearing at 2.8 minutes (peak 7). Further digestion leads to more Cy3-asp-arg-val-tyr formation (peak 7) and its degradation product, Cy3-asp-arg-val (peak 8). A new 2 species, Cy3-asp-arg (peak 9) begins to show up, as seen in Figure 2C. (Note: the structural assignments were made based on the electrophoretic mobilities of the species). Further digestion (124 min, Figure 2D) results in the disappearance of peak 7 while peak 9 continues to grow at the expense of peak 8. At 310 min (Figure 2E), peak 9 is the only one remaining. The structure representing peak 9 is identical to the product of the tryptic digestion of Cy3-angiotensin I and Cy3-angiotensin II, shown in Figure 1B. Addition of the enzyme carboxypeptidase P to the digest of E yields Cy3-asp (Figure 2F) identical to the product (peak 10) obtained in Figure 1C.

Digestion of Cy3-Angiotensin I with Carboxypeptidase Y
Carboxypeptidase Y is a serine protease which successively releases all amino acids from the C-terminal of peptides or proteins. One minute after the addition of the carboxypeptidase Y to a 10 nM solution of Cy3-angiotensin I, the formation of four major species can be observed, i.e., Cy3-asp-arg-val-tyr-ile-his-pro-phe (peak 3, Cy3-angiotensin II), Cy3-asp-arg-val-tyr-ile-his (peak 5), Cy3-asp-arg-val-tyr (peak 7), and Cy3-asp-arg-val (peak 8).

Figure 2. A: CE-LIF electropherogram of Cy3-angiotensin I, 20 nM, and Cy3 diacid, 1.0 nM. LIF source: same as that in Figure 1. B: Proteinase K digest of Cy3-angiotensin I at 8 min. C: Digest at 102 min. D: Digest at 124 min. E: Digest at 310 min. F: Carboxypeptidase P digest of the reaction mixture of Figure 2E. Peak i.d.: see Table 1; run voltage: 20 kV.

Figure 3. A: CE-LIF electropherogram of Cy3-angiotensin I, 20 nM, and Cy3 diacid, 1.0 nM. Laser light source: A “green” helium-neon laser, emitting at 543 nm (Particle Measurement Systems). Peak i.d.: see Table 1; run voltage: 20 kV. B: Carboxypeptidase Y digest of Cy3-angiotensin I at 1.0 min. C: Digest at 20 min. D: Digest at 60 min. E: Digest at 250 min. F: Addition of proteinase K to the reaction mixture of E.
Minor species are Cy3-asp-arg-val-tyr-ile-his-prophe-his (peak 2), Cy3-asp-arg-val-tyr-ile (peak 4), and Cy3-asp-arg-tyr-ile (peak 6), shown in Figure 3B. Each of the species is the result of sequential hydrolysis from the C-terminal end of the peptide. Continued digestion after 20 min leads to the accumulation of two major species, peaks 7 and 8 (Figure 3C). Further digestion yields peak 8 at the expense of 7 (Figure 3D). Exhaustive digestion results in the formation of peak 8, Cy3-asp-arg-val, as the end product (Figure 3E). Addition of proteinase K to the Cy3-asp-arg-val (peak 8) leads to the formation of Cy3-asp-arg, Figure 3F, identical to the product of trypsin-catalyzed digestion of Cy3-angiotensin I and II shown in Figure 2B.

**Conclusion**

Protease-catalyzed hydrolysis of a substrate can be studied by CE. Labeling the substrate with the cyanine dye Cy3 permits highly sensitive PACE-LIF detection in conjunction with economical laser light sources. The CE technique provides high-resolution separation and (potential quantitation) of substrate and digestion products. Furthermore, the dynamic range of LIF detection is relatively large, typically 5–6 orders of magnitude. This should allow, for example, routine detection of $10^{-9}$ M product in the presence of $10^{-6}$ M substrate. The present method can be readily adapted by employing well-defined peptide sequences to probe the specificity of proteases in real samples (e.g., fermentation broths, serum). Kinetics of relative hydrolytic reactivity can also be studied with a similar approach. The concept should also be applicable to monitoring the activity of other types of enzymes such as synthetases.

<table>
<thead>
<tr>
<th>Table 1. Peak Identification of Figures 1–3.</th>
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<tr>
<td>1. Cy3-asp-arg-val-tyr-ile-his-prophe-his-leu (Cy3-angiotensin I)</td>
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<td>3. Cy3-asp-arg-val-tyr-ile-his-prophe (Cy3-an-giotensin II)</td>
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**References:**