Electrochemical Oxidative Cleavage of Peptides

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Table of Contents

Introduction

Studying life is an immense scientific endeavor. Interactions among DNA, RNA, and proteins lie at the heart of the complex systems used by living organisms. Understanding the nature of the interaction among and within these different biomolecules is an area of significant research. Proteomics, the study of proteins, has become the focus of many research projects.

Common techniques of proteomic analyses employ the use of enzymatic and chemical digestion reagents. For example, the enzyme trypsin is used to digest proteins where cleavage occurs at the basic arginine and lysine amino acids. Some new developments in the field of protein analysis employ exotic techniques. Permentier and colleagues published work describing an electrochemical oxidative cleavage that proceeds through a two-electron reduction followed by hydrolysis at tyrosine in a peptide chain.[1,2] High-intensity focused ultrasound was used by Lopez-Ferrer et al. to cause proteolytic cleavage within seconds.[3] Exploring the electrochemistry of amino acids and applying the information to peptide cleavage is the focus of this research.

Electrochemical cleavage of a protein chain would be more advantageous than enzymatic methods because successful implementation of this technology would reduce or eliminate the need to use enzymes, which can be very expensive. Time scales would be shorter and robust chemical environments could be employed to effect the desired oxidative cleavage under varied environmental conditions, like extraterrestrial or deep sea confines. Furthermore, it could be tunable so that selection of the cleavage sites within a sequence can be varied rather than being limited to only those sites that react with a

digestion reagent. In addition to these advantages, using electrochemical methods would provide greater insight into the overall nature of the electron-transfer processes of proteins.

Experimental Techniques and Procedures

Reagents

Glycine was supplied by EM Science, and all other amino acids and N-acetyl-L-tryptophanamide were supplied by Sigma Aldrich. Purity of the amino acids was >97%. Ammonium bicarbonate was purchased from Mallinckrodt. Phosphate buffers, potassium chloride, and potassium ferricyanide were purchased from EM-Science. Peptides were provided by the DiMarchi research group. Ultrahigh purity argon (UHP Ar) was supplied by Matheson Tri-gas. Water used was supplied by the in-house distilled water supply system unless otherwise noted.

Electrodes, Electrochemical Cells, and Potentiometers

Working electrodes were constructed in house and consisted of a core of working electrode material pressed into a Teflon® sleeve. Polishing of the working electrodes was accomplished by abrasion of the surface in a circular pattern for 45-60 s with a 0.05-μm alumina slurry on a polishing pad. After the polishing, the working electrode was rinsed with water, dipped into a clean ultrasonic water bath, and rinsed again. The reference electrode was a saturated calomel electrode (SCE) having a potential of +0.242 V vs. the

standard hydrogen electrode (SHE). All potentials are referenced to this value. Auxiliary electrodes were constructed of a Pt coil soldered to a Cu wire.

Described elsewhere are designs of the electrochemical cells used for cyclic voltammetry (CV)[4] and controlled-potential electrolysis (CPE).[5] Other electrochemical designs that were employed will be described later in this paper.

CV data were collected with the aid of either an in-house three-electrode potentiostat connected to a computer with internally written software or an Obbligato Objectives Faraday MP® commercial potentiostat that used Faraday MP Version 1.5 software. CPEs were conducted with the Faraday MP Potentiostat.

Solutions were sparged with UHP Ar for at least 15 min to remove dissolved oxygen prior to analysis.

Additional Instrumentation

High-performance liquid chromatographic (HPLC) analyses with ultraviolet (UV) and mass spectrometric (MS) detection were performed either by the DiMarchi research group or by the Indiana University (IU) Mass Spectrometry Facility. Analyses of the gold nanoparticle samples were performed with a narrow-bore column, 10 cm × 0.5 mm with 5-µm particle size and 300-Å pores, fabricated with the assistance of the Reilly research group.

Dynamic light scattering (DLS) analysis of the gold nanoparticles was conducted in the IU Physical Biochemistry Facility with a Malvern Instruments Zetasizer® Nano Series DLS equipped with a 633-nm laser. Tunneling electron microscopy (TEM) was performed by the Dragnea research group.

Results and Discussion

Initial work was done to reproduce CV results that Reynaud et al. obtained for cysteine.[6] Figure 1 shows example CVs of L-cysteine at Pt, Au, and glassy carbon working electrodes. Results were in good agreement with the literature.

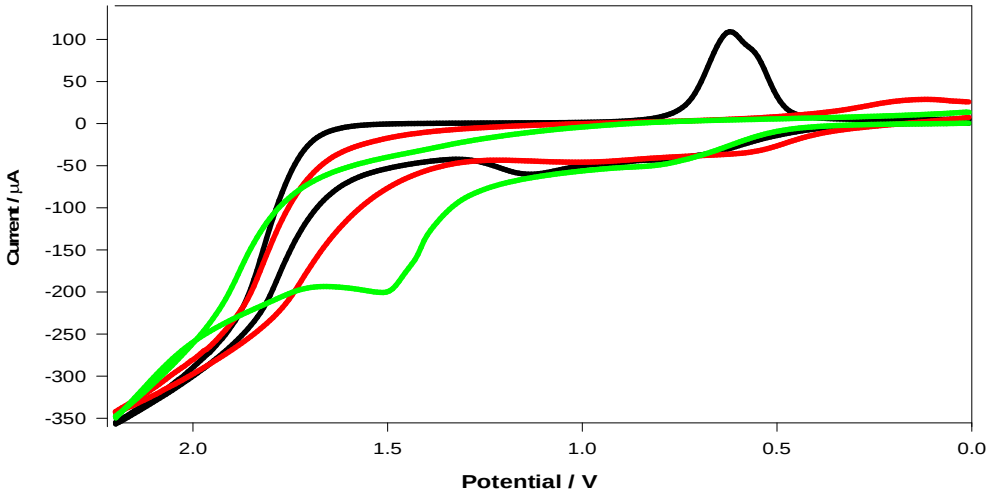

Figure 1. Cyclic voltammograms for 3.48 mM L-cysteine collected in pH 6.2 phosphate buffer (57 mM KH_2PO_4 and 14 mM K_2HPO_4) at various working electrodes: glassy carbon (green), gold (red), and platinum (black); auxiliary electrode, Pt coil; reference electrode, SCE; scan rate 100 mV/s; potentials swept from 0.0 to +2.0 to 0.0 V.

Two cathodic peaks at E_{pc} = +0.6 V and +0.55 V (shoulder) were seen when a platinum electrode was used. Small oxidation waves were seen with both the gold and platinum electrode, but glassy carbon showed the greatest anodic peak current (E_{pa}) with a maximum value of –85 A at +1.5 V. Anodic currents are more desirable for this project as they lead to oxidation and eventual cleavage of bonds.

CV data from the glassy carbon electrode compare favorably with the voltammogram recorded with a vitreous carbon electrode seen in the literature.[6] Differences between data collected with a glassy carbon electrode versus a vitreous carbon electrode stem from the different scan rates at which the data were collected. Current scales as the square-root of scan rate. Reynaud's CV was collected at 5 mV/s, whereas those in Figure 1 were collected at 100 mV/s. Increasing the scan rate by a factor of 20 increased the resultant currents by a factor of 4.5.

After it was established that anodic current can be generated for L-cysteine at a glassy carbon electrode, refinements in the experimental design were made to ensure compatibility with subsequent analytical techniques for obtaining structural information. One possible complementary analytical procedure is matrix-assisted laser desorption-ionization (MALDI). Because Na^+ and K^+ adducts readily form in MALDI, it was necessary to find an alternative supporting electrolyte that did not contain either of these species. Changing the supporting electrolyte from potassium phosphate buffer to ammonium carbonate buffer would allow for the use of MALDI while maintaining control over the pH of the systems tested. Table 1 summarizes the results of some CV experiments with the new buffer system and some additional amino acids. CVs collected with a glassy carbon working electrode showed anodic peaks for each of the amino acids tested, whereas Pt and Au working electrodes did not.

Table 1. Summary of CV experiments using NH_4HCO_3 buffer as the supporting electrolyte. Working electrode listed in the table; reference electrode, SCE; auxiliary electrode, Pt coil; scan rate 100 mV/s; supporting electrolyte 50 mM NH_4HCO_3 buffer pH = 8.2. None detected (nd) is listed when no anodic current above background was seen.

Amino Acid	Working Electrode Material Potential of Peak Anodic Current (V)		
	Au	Pt	Glassy C
Cysteine	+1.0	+0.8	+1.5
Methionine	+1.1	+1.3	+1.6
Glycine	nd	nd	+1.4

VQAAIDYING was obtained from the DiMarchi research group at IU. This peptide was already in stock in the DiMarchi storage facility and contained a single tyrosine residue which is similar to the peptide that had been previously analyzed.[1] Characterization of this pentapeptide was performed by David Smiley with the aid of HPLC-UV and MS. HPLC purity analysis at 215 nm showed the pentapeptide to be 62% of the major constituent based on area percent calculations. MS analysis showed the major constituent to have a mass of 1062 D which is consistent with the proposed sequence. Because the pentapeptide was synthesized in house, this analytical information was deemed as sufficient verification of the peptide sequence and purity.

CVs of VQAAIDYING at Pt, Au, and glassy carbon electrodes were obtained and examples are overlaid in Figure 2. Cyclic voltammograms collected with the Pt working electrode showed small anodic and cathodic currents as compared to the background level. Peak potentials, E_{pa} and E_{pc}, are at +0.78 V and –0.17 V, respectively. Cyclic voltammograms recorded with a Au working electrode showed a large cathodic current at +0.25 V. No significant anodic peaks were observed with the Au working electrode.

Figure 2 clearly displays the comparatively large anodic current at E_{pa} of +0.52 V when glassy carbon is used. Therefore, glassy carbon was selected as the working electrode material for subsequent tests.

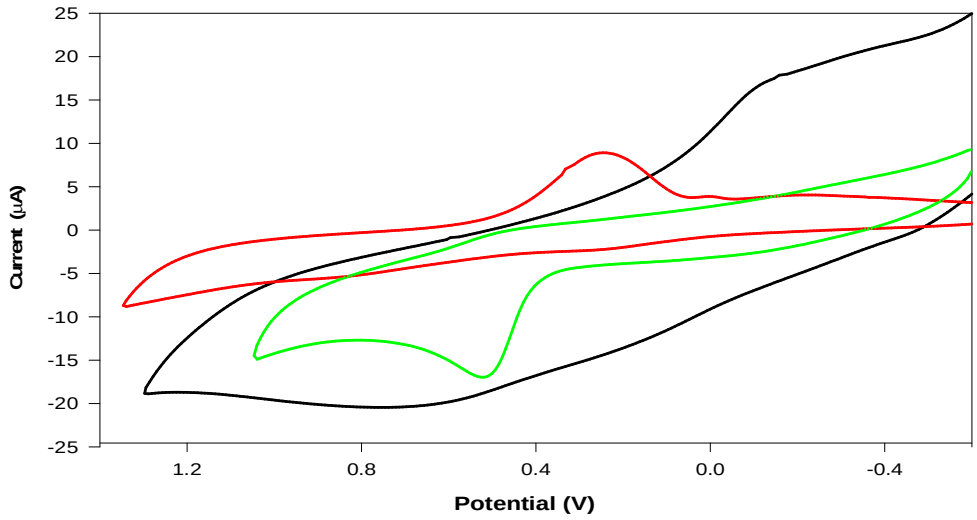

Figure 2. Cyclic voltammograms for 1.84 mM VQAAIDYING in 50 mM NH_4HCO_3 at Pt (black), Au (red), and glassy C (green) working electrodes; reference electrode, SCE; auxiliary electrode, Pt coil; scan rate 100 mV/s; potentials sweep from more negative to positive to negative.

Follow-up experimentation requires oxidation of the peptide to determine if bond cleavage can be induced by means of this set of parameters. Elucidation of the mechanism involved in bond cleavage will also necessitate counting how many electrons are transferred in the reaction. Thin-layer electrochemistry is particularly suited for this purpose because complete oxidation of a sample can be done very quickly. Additionally, in thin-layer systems the volume of sample is small which would be well suited for analysis of biological systems where amounts will be limited. Design of a suitable thin-layer electrochemical cell employing a glassy carbon electrode was begun.

Shaeffer's design for a simple thin-layer electrochemical cell[7] was used as a starting point in the development of another system. One can fabricate a relatively simple thin-layer electrode by inserting a Pt wire into a Teflon® sleeve that is press-fitted into a glass capillary. Shaeffer's design used an "L" shaped piece of Pt wire as the electrode. Because no such shape of glassy carbon was readily available, modifications were needed.

A 3-mm-diameter glassy carbon rod was inserted into a Teflon® sleeve with an inner diameter only just slightly larger than that of the rod. Space between the carbon rod and the wall of the sleeve fills via capillary action and the dimensions would be sufficient for execution of thin-layer experiments.

Construction of this cell was completed with the aid of equipment in the IU student machine shop. Potassium ferricyanide was used as a standard to test the cell, because its reversible one-electron redox couple is well characterized. Figure 4 is an example CV obtained with the cell design.

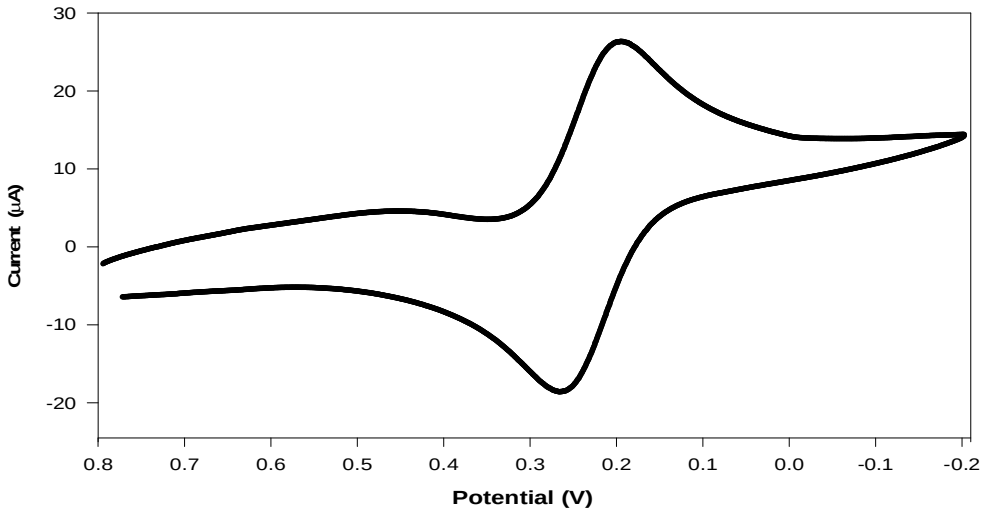

Figure 3. Cyclic voltammogram for 4.4 mM $K_3Fe(CN)_6$ in 1 M KCl; reference electrode, SCE; auxiliary electrode, Pt coil; working electrode, glassy C; scan rate 10 mV/s; potentials swept from +0.8 to –0.2 to +0.8 V.

Cathodic and anodic peaks occurred at E_{pc} of +0.195 V and E_{pa} of +0.266 V. From these values, E^0 calculates as +0.230 V with a peak separation of 71 mV. Literature reduction potential of the $[Fe(CN)_6]^{3-} + e^- \; D \; [Fe(CN)_6]^{4-}$ couple is given as +0.116 V vs. SCE (+0.358 vs. SHE), which compares poorly with the reduction potential measured in this experiment. Differences between theoretical and experimental data were likely due to uncompensated resistance within the cell. Large distances between the electrodes leads to increased IR drop and exacerbates potential-gradient formation. In a thin-layer cell the potential difference between cathodic and anodic peaks should be close to zero. Another feature of the CV is the shape of the peaks. Ideal behavior predicts a Gaussian shape for CV peaks in a thin-layer electrochemical cell. Also, because the end of the glassy carbon rod was exposed to the bulk solution, the CV looks typical for a standard diffusion-controlled system. Combining this information led to the conclusion that a different cell would be necessary to obtain the thin-layer characteristics needed to accomplish the task of measuring the number of electrons transferred in the desired oxidation.

Once again, student-machine-shop tools were employed to build a device that would hopefully be capable of aligning electrodes in a thin-layer electrochemical cell in a reproducible manner. Aluminum was machined to form a support for a micrometer to which was attached a glassy carbon rod. Figure 4 is a schematic diagram of the Al block support device.

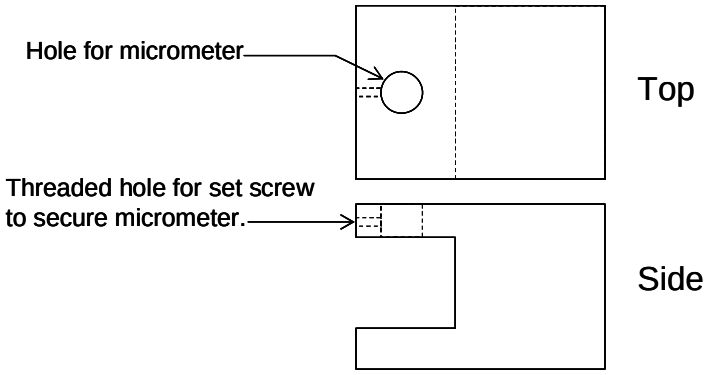

Figure 4. Aluminum block to hold micrometer in place to control layer thickness.

Glassy carbon was attached to the end of a micrometer with a press-fit brass collar. Anchoring the micrometer in the support block was accomplished with a set screw. The glassy carbon end of the micrometer served as the working electrode and the micrometer was used to control the cell volume. Potassium ferricyanide served as the standard to evaluate this new set-up.

CVs of a 10.57 mM $K_3Fe(CN)_6$ standard solution did not exhibit the thin-layer characteristics desired. Peak shapes were not Gaussian, and E_{pc} was separated from E_{pa} by an average of 35 mV. More detrimental to the hopes for use of this design was the large difference in current values between analyses. Cathodic peak currents ranged from −16 to −27 µA, and anodic peak currents ranged from 16 to 25 µA. Such large differences in peak currents indicate that the cell did not have a reproducible layer thickness. Design of a viable thin-layer cell is still underway; however, a new approach to electrochemical cleavage was considered.

Many electrochemical investigations of amino acids and proteins have been conducted with some form of mediator.[8-12] Alternatively, Rana and Meares used an iron chelate to effect cleavage of a peptide.[13] Elemental gold and sulfide containing

compounds are known to interact readily at room temperature. Could a suspension of Au nanoparticles bind S-containing amino acid residues in a protein and make a convenient bridge through which oxidative cleavage could be induced?

Au nanoparticles were synthesized from a solution of chloroauric acid ($HAuCl_4$) according to the citrate method. Characterization of the nanoparticles was performed by means of DLS and TEM. Measurements of the particles revealed that they were 53 (±10) nm in diameter (Figure 5).

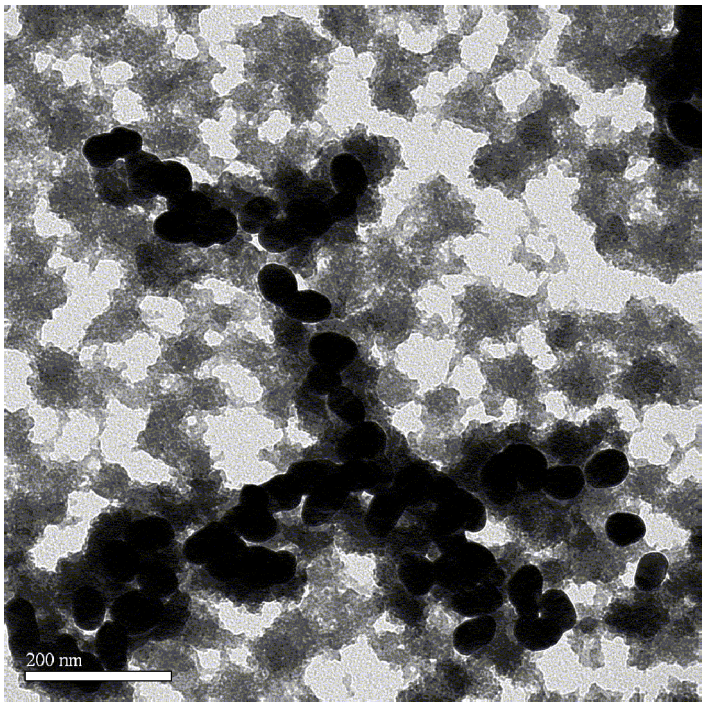

Figure 5. TEM image of Au nanoparticles.

After consultation with Jay Levy of the DiMarchi research group and Jon Karty of the IU Mass Spectrometry facility, it was decided that a small peptide with the sequence FAAACGGGF would be well suited for experiments using the Au nanoparticles. Having one central sulfur-bearing amino acid and different side chains terminated by UV absorbing groups, this sequence upon cleavage would yield easily identified products.

Evaluating the merit of this idea began with CV analysis of cysteine in the presence of varying amounts of Au nanoparticles (Figure 6).

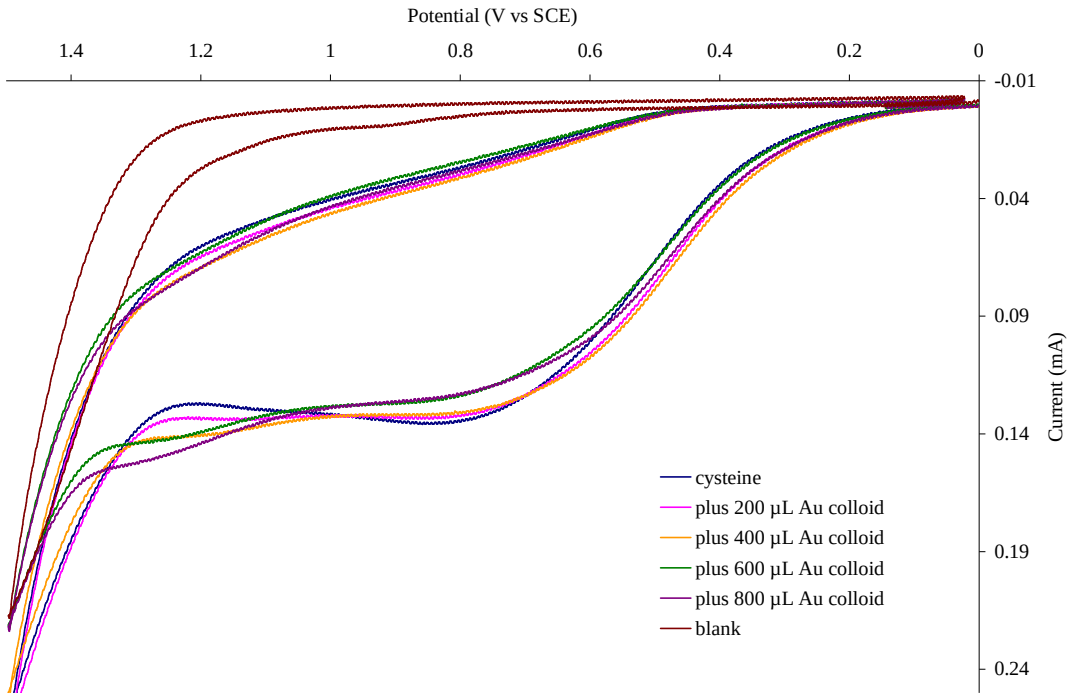

Figure 6. Cyclic voltammograms for 14.7 mM cysteine in 62.5 mM NH_4HCO_3 with increasing amounts of ~1 µM Au nanoparticle suspension; working electrode, glassy carbon; reference electrode, SCE; auxiliary electrode, Pt coil; scan rate, 100 mV/s; potentials swept from 0 to +1.5 to 0 V; 43–63 nm Au nanoparticle size.

Cysteine analyzed in the absence of the Au nanoparticles showed an E_{pa} at +0.83 V. Addition of the nanoparticles resulted in a decrease of current at +0.83 V and an increase in current at +1.25 V. As the thiol group in cysteine adsorbs to the surface of a Au nanoparticle the electrochemical behavior of the gold becomes dominant. When the peptide binds to a conductor (Au) it was hoped that electrons could be readily extracted from the bound molecule.

CPE of the test peptide FAAACGGGF was conducted in a Klein cell[5] with the Au nanoparticles added. HPLC analysis of the pre- and post-electrolysis solutions showed no significant differences (Figure 7). Use of the Au nanoparticles to effect cleavage of bonds within the peptide was unsuccessful.

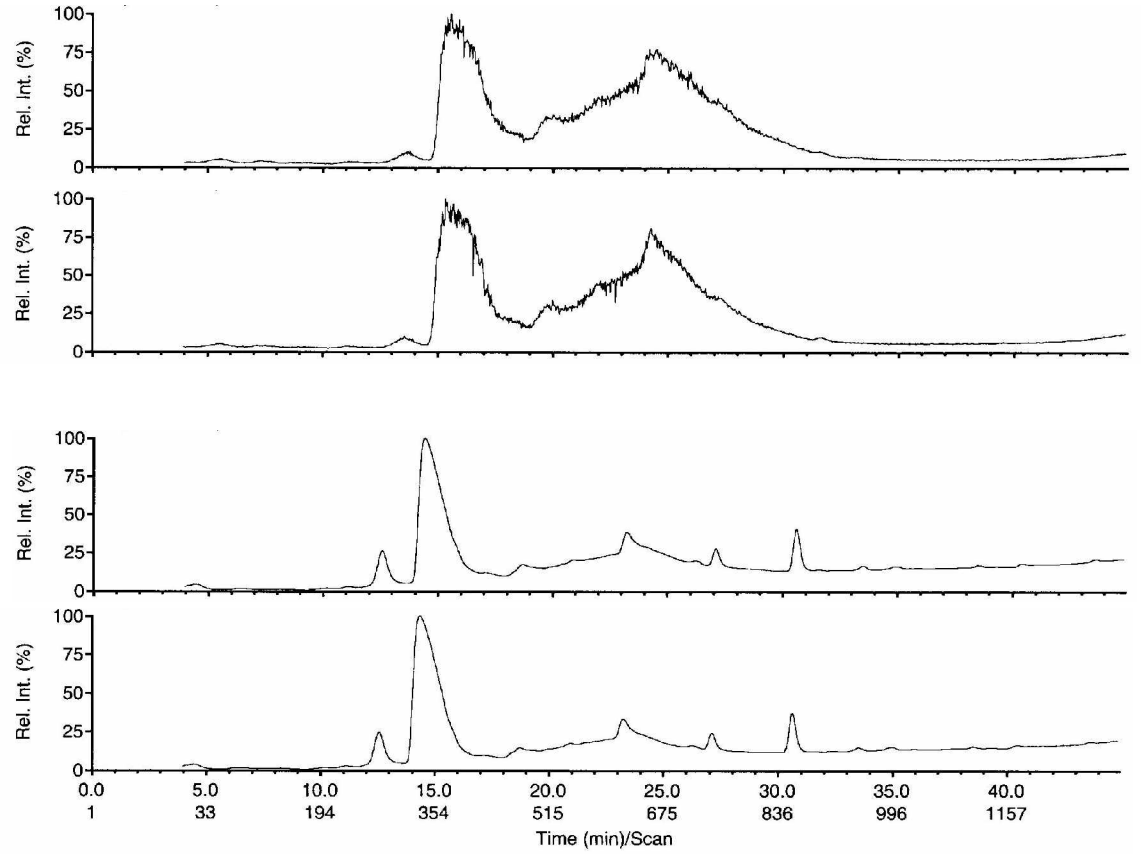

Figure 7. HPLC-MS (top two) and HPLC-UV (bottom two) of the test system after CPE (above) and before CPE (below) for each detection strategy employed.

Design of an electrochemical system to accomplish the task of proteolytic peptide cleavage is not a trivial task. While work conducted so far includes evaluation of amino acids at various working electrode materials, development of several thin-layer electrochemical cell designs, and exploration of Au nanoparticle-mediated electrochemical oxidation, a great deal more work is necessary to accomplish the task. Primarily,

conditions where oxidation of the bonds within a protein backbone sufficient to cause cleavage need to be determined.

Acknowledgements

Thanks to all of the people who made this research project such an enjoyable and edifying experience. Brendan Sweeny provided the initial training on the use of the in-house built potentiostat and data collection system. Amino acids obtained from the Reilly group made it easy to conduct experiments without having to order them and wait for their shipment to arrive. Xiaohui Liu helped me build a micro-bore column for the HPLC analysis of the Au nanoparticle-containing samples. Dave Smiley and Jay Levy of the DiMarchi research group assisted by supplying and characterizing the test peptides. Dr. Marie-Christine Daniel conducted the TEM measurements and provided guidance in synthesizing the nanoparticles. Other members of the Peters research group provided support and education for the operation of the laboratory equipment, especially Peng Du and Bianna Smith. Finally, thank you Dr. Dennis G. Peters for accepting me into your group and allowing me to attempt this very difficult task.

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