Cystine, an Essential Determinant of Protein Tertiary Structure, Is Also a Target for Electrochemical Manipulation

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Overview

Purpose: Development of UHPLC and HPLC-electrochemical (EC) detection methods for the measurement of different cystine-containing peptides including simple peptides (glutathione), medium sized peptides (insulin), cyclic peptides (oxytocin and vasopressin) and peptides with cystine knots (cyclotides).

Methods: UHPLC- and HPLC-based methods using electrochemical detection with a robust boron-doped diamond (BDD) working electrode are described.

Results: The novel BDD working electrode extends the range of analytes capable of being measured by EC detection, making measurement of low levels of peptides possible.

Introduction

The ability for thiol residues to undergo oxidation to disulfides is biochemically important, since it affects antioxidant activity (e.g., glutathione) and determination of protein tertiary structure. The formation of cystine disulfide bridges from two cysteine residues not only influences protein confirmation but also renders it electrochemically active. Although peptides can be quantified by electrochemical oxidation of the disulfide bond, the commonly used working electrode, glassy carbon, suffers from two major issues – a limited oxidation potential and the persistent fouling of the electrode’s surface necessitating frequent cleaning procedures. The boron-doped diamond (BDD) working electrode, on the other hand, can be used at extreme oxidation potentials and, as its surface is inert, does not suffer from adsorption problems. The ability for HPLC with electrochemical detection to quantify a variety of disulfide containing peptides was evaluated and included measurement of GSH and glutathione disulfide (GSSG), cyclic nonapeptides (vasopressin and oxytocin), an example of intra and inter chain disulfide cross-linking (insulin) and a cyclic, disulfide rich cyclotide (cycloviocin O2) (Figure 1).

Figure 1. Structures of peptides used during method development.
Methods

HPLC System: Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLC system including:
- HPG-3600RS pump
- WPS-3000TRS autosampler
- 3000 TCC-3000RS column thermostat
- DAD-3000RS diode array detector
- ECD-3000RS electrochemical detector with 6041RS ultra Analytical Cell and boron doped diamond (BDD) electrode

1) Analysis Of Glutathione Redox Status by UHPLC-EC Detection

Column: Thermo Scientific™ Accucore™ RP-MS column, 2.6 μm, 2.1 × 150 mm
Pump Flow Rate: 0.500 mL/min
Mobile Phase: 0.1% pentafluoropropionic acid, 0.02% ammonium hydroxide, 2.5% acetonitrile, 97.4% water
Column Temperature: 50.0 ºC
Post Column Temperature: 25.0 ºC
Injection Volume: 2 μL standards; 4 μL samples
Cell Potential: 1600 mV
Filter Constant: 1.0 s
Cell Clean: On
Cell Clean Potential: 1900 mV
Cell Clean Duration: 10.0 s

2) Analysis Of Oxytocin, Vasopressin and Insulin by HPLC-EC Detection

Column: Accucore phenyl hexyl column, 2.6 μm, 2.1 × 100 mm
Pump Flow Rate: 0.600 mL/min
Mobile Phase: A: 50mM lithium perchlorate, 0.05% TFA in water
          B: 50mM lithium perchlorate, 0.05% TFA in acetonitrile
Gradient: 15 to 50% B in 10min
Column Temperature: 35.0 ºC
Injection volume: 5 μL
Cell Potential: 1800 mV
Filter Constant: 1.0 s
Cell Clean: On
Cell Clean Potential: 1950 mV
Cell Clean Duration: 20.0 s

3) Analysis Of Cyclotides by HPLC-EC Detection

Column: Thermo Scientific™ Acclaim™ C18  2.2 μm, 3 x 75mm
Pump Flow Rate: 0.500 mL/min
Mobile Phase: A: 50mM ammonium formate in water, pH4.4
          B: 65% acetonitrile, 15% water, 20% 100mM ammonium formate at pH4.4
Column Temperature: 45.0 ºC
Gradient: 0-15min, 30-100%B; 15-20min, 100%B
Injection Volume: 10 μL standards
Cell Potential: 1500 mV
Cell Clean Potential: 1900 mV
Cell Clean Duration: 10.0 s
Results

1) Analysis Of Glutathione Redox Status

An instrumental prerequisite for trace analysis is that the HPLC system must be inert (free from leachable transition metals) in order to achieve optimal sensitivity using an electrochemical detector. The system uses biocompatible materials in the flow path to reduce the influence of metal that contribute to elevated background currents at the electrochemical cell. Use of the Thermo Scientific Dionex 6041RS ultra Analytical Cell provides the unique electrochemical capabilities of the boron-doped diamond which enables the oxidation of organic compounds using higher electrode potentials than other working electrode materials.

The direct electrochemical detection of aminothiol compounds only using a boron-doped diamond electrochemical cell has been previously described. The applied potential used for this study (+1600 mV) was sufficient to oxidize both thiol and disulfide analytes. Advantages of this approach include a stable electrode surface and method simplicity since no sample derivatization is required. After each analysis the electrode surface was regenerated by a 10 second clean cell pulse at +1900 mV. After a short re-equilibration period the electrode was once again stable and could be used for the analysis of the next sample. Figure 2 illustrates the overlay of calibration standards for these compounds ranging from 1–20 µg/mL. Excellent peak resolution and retention time uniformity were observed. The column used for this method was the Accucore RP-MS 2.6 micron solid-core material which provides fast, high resolution separations but with lower system pressures.

Figure 2. Overlay of GSH, GSSG and related analytical standards ranging from 1–20 µg/mL (CysGly – cysteinyl-glycine; HCYS – homocysteine).

Good linearity of response to different concentrations was obtained with correlation coefficients ranging from \( R^2 = 0.989–1.00 \) for the five compounds evaluated (Table 1) over the range of 1–20 µg/mL. The percent relative standard deviation (%RSD) for calibration curves (five concentrations in triplicate) is shown.

Table 1. Regression data for standard calibration curves.

<table>
<thead>
<tr>
<th>Peak</th>
<th># Points</th>
<th>RSD %</th>
<th>Correlation Coefficient</th>
<th>Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>CysGly</td>
<td>15</td>
<td>8.9059</td>
<td>0.989</td>
<td>0.0481</td>
</tr>
<tr>
<td>GSH</td>
<td>15</td>
<td>1.7446</td>
<td>1.00</td>
<td>0.0509</td>
</tr>
<tr>
<td>Meth</td>
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<td>3.4526</td>
<td>0.999</td>
<td>0.0736</td>
</tr>
<tr>
<td>HCYS</td>
<td>15</td>
<td>1.9282</td>
<td>1.00</td>
<td>0.0268</td>
</tr>
<tr>
<td>GSSG</td>
<td>15</td>
<td>1.2458</td>
<td>1.00</td>
<td>0.0170</td>
</tr>
</tbody>
</table>
2) Analysis Of Oxytocin, Vasopressin and Insulin

The ability to measure more complex cystine-containing peptides and proteins using HPLC with electrochemical detection on a BDD working electrode was then evaluated. Two cyclic peptide hormones formed by a cystine bridge (oxytocin and vasopressin), and a more complex protein hormone having both inter- and intra-chain cystine linkages (insulin), were chosen. Chromatographic separation of all three compounds on a phenyl hexyl column is presented in Figure 3. Figure 4 shows HDV of the three peptides. The estimated limit of detection was 100-200 pg on column for both vasopressin and oxytocin. The ability to measure GSH and GSSG (above) shows that oxidation of both thiols and disulfides can contribute to the overall electrochemical signal obtained from these peptides. In the case of oxytocin and vasopressin the contribution from the oxidation of cystine maybe augmented by other residues that can undergo oxidation (e.g., each contains a tyrosine residue). The electrochemical behavior of insulin is far more complex. Contribution to the overall response will come from oxidation of the three cystine residues, but each polypeptide chain has two tyrosine residues that can also respond. Although insulin is a small protein (51 residues) its ability to oxidize will be dependent upon interaction between its resides and the BDD working electrode surface. Kinetic hindrance will result in a decreased signal (proportional to 1/MW).

Figure 3. Chromatogram showing detection of 8-vasopression, oxytocin and insulin electrochemically with boron doped diamond electrode at 1800mV.

Figure 4. HDV of vasopressin, oxytocin and insulin on a BDD electrode.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Calibration Curve</th>
<th>Limit of Detection (pg)</th>
<th>Area Response (nA.min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vasopressin</td>
<td></td>
<td></td>
<td>0 to 200</td>
</tr>
<tr>
<td>Oxytocin</td>
<td></td>
<td></td>
<td>0 to 200</td>
</tr>
<tr>
<td>Insulin</td>
<td></td>
<td></td>
<td>0 to 200</td>
</tr>
</tbody>
</table>

Table 1. Regression data for standard calibration curves.
3) Analysis of Cyclotide by HPLC-EC detection
Cyclotides are small disulfide rich peptides containing 28-37 amino acid residues and are isolated from plants. The tertiary structures of these peptides are very interesting as the three cystine bridges form a cystine knot motif (Figure 1) imparting great stability to the peptide. Today, over 100 cyclotides have been isolated and characterized from a number of species including the Rubiaceae, Violaceae, and Cucurbitaceae. In the plant cyclotides act as both natural insecticides and anti-microbial agents. The cyclotide structure is of interest to pharmaceutical companies for the development of potential anti-HIV and anti-tumor agents. For these studies, the cyclotide cycloviolacin O2, a 30 residue cyclic peptide found in the sweet violet (Viola odorata) was studied. Figure 5 shows the detection of cycloviolacin O2 both as the standard and its presence in a crude extract of the sweet violet.

Conclusion
- UHPLC/HPLC separation and detection on a BDD working electrode is a practical approach to the measurement of cystine-containing peptides and proteins.
- The BDD working electrode overcomes the practical limitations of traditional carbon working electrodes.
- Future work will a) evaluate the contribution of other amino acid residues to the overall response of the peptide/protein and b) the inclusion of a BDD working electrode prior to the electrospray source of a mass spectrometer to study the mechanism of peptide/protein oxidation and possible cleavage reactions.

Acknowledgements
The standard of cycloviolacin O2 and the crude extract of the sweet violet were kindly provided by Professor Ulf Göransson from Uppsala University in Sweden.

References