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Electrochemical Protein Cleavage in a Microfluidic Cell with Integrated Boron Doped Diamond Electrodes

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S Supporting Information

ABSTRACT: Specific electrochemical cleavage of peptide bonds at the C-terminal side of tyrosine and tryptophan generates peptides amenable to liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis for protein identification. To this end we developed a microfluidic electrochemical cell of 160 nL volume that combines a cell geometry optimized for a high electrochemical conversion efficiency (>95%) with an integrated boron doped diamond (BDD) working electrode offering a wide potential window in aqueous solution and reduced adsorption of peptides and proteins. Efficient cleavage of the proteins bovine insulin and chicken egg white lysozyme was observed at 4 out of 4 and 7 out of 9 of the predicted cleavage sites, respectively. Chicken egg white lysozyme was identified based on 5 electrochemically generated peptides using a proteomics database searching algorithm. These results show that electrochemical peptide bond cleavage in a microfluidic cell is a novel, fully instrumental approach toward protein analysis and eventually proteomics studies in conjunction with mass spectrometry.

The study of protein structures and interactions is key to the understanding of biological functions such as those related to health and the development of disease.¹ The large number of different species in the human proteome makes it particularly difficult to accurately identify and quantify proteins in complex biological mixtures. Various methods based on liquid chromatography coupled to mass spectrometry (MS) have been developed to analyze complex protein mixtures from biological samples in a comprehensive manner.^{2,3}

Cleaving proteins into defined peptide fragments is currently the main approach, often referred to as bottom-up proteomics. Information about the proteins, originally present in the sample, can be obtained in various ways, for example, by peptide mass fingerprinting⁴ or tandem MS (MS/MS) after chromatographic separation of the generated peptides. To this end, experimental MS/MS spectra are compared to *in silico* generated spectra from protein sequence databases.⁵ More challenging but of increasing importance is direct sequence analysis of the generated peptide fragments (*de novo* sequencing) without resorting to databases as well as the comparison of MS/MS spectra to an increasing number of spectral libraries.^{6–8}

Highly specific cleavage of peptide bonds is critical for bottom-up proteomics. Enzymatic digestion using proteases is the most widespread method for cleavage of proteins at specific peptide bonds, and a number of proteases with different

specificities are available. The most commonly used protease is the enzyme trypsin, which cleaves specifically at the C-terminal side of lysine and arginine (except if followed by a proline).⁹ Alternatively, chemical cleavage may be used if specificity for a certain peptide bond or amino acid sequence is required for which no protease is available.⁹

Electrochemical protein cleavage is emerging as an instrumental alternative to chemical and enzymatic approaches. Over 50 years ago it was found that peptide bonds can be cleaved electrochemically at the C-terminal side of tyrosine.¹⁰ Later, electrochemical peptide bond cleavage at the C-terminal side of tryptophan was also described.¹¹ Compared to chemical and enzymatic protein cleavage, the electrochemical approach offers advantages of (1) being a rapid, purely instrumental approach that does not require additional reagents, (2) having a cleavage site specificity for which no enzymatic or chemical cleavage reagent is available, (3) opening the possibility to cleave proteins under conditions (e.g., strongly denaturing) that enzymes cannot tolerate, and (4) providing the possibility to label reactive groups that are uniquely generated upon electrochemical cleavage.

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It appeared early on that the choice of electrode material is crucial, as illustrated by electrochemical oxidation and cleavage of tyrosine- and tryptophan-containing dipeptides at platinum electrodes, where strong adsorption was observed.¹² Electrochemical peptide bond cleavage continued to be investigated using flow-through (coulometric) cells with porous graphite electrodes. Coupling these cells online to electrospray ionization mass spectrometry (ESI-MS) resulted in a system for rapid peptide analysis capable of tyrosine-specific oxidation and peptide bond cleavage, as demonstrated with a variety of peptides.¹³ Following these investigations, the oxidation and cleavage mechanisms of tyrosine- and tryptophan-containing tripeptides were studied in detail.¹⁴ Main challenges were that cleavage yields were limited due to competing oxidation reactions and recoveries were hampered due to adsorption, as exemplified for the tryptophan-containing decapeptide adrenocorticotrophic hormone (ACTH) (1-10).¹⁵ Despite these initial shortcomings, electrochemical peptide bond cleavage was successfully combined with liquid chromatography in an EC-LC-MS/MS setup for protein analysis.¹⁵ Problems with adsorption at the electrode surface and limited cleavage yields were aggravated in the case of proteins, requiring extensive regeneration of the porous graphite electrode surface and careful optimization of the reaction conditions to observe peptide bond cleavage.

Electrochemical peptide bond cleavage was subsequently achieved in thin-layer (amperometric) flow cells with glassy carbon (GC) and boron doped diamond (BDD) electrodes.¹⁶ Notably, BDD electrodes performed better in terms of lower adsorption, resulting in higher cleavage yields due to both a better recovery of products and a lower degree of dimer formation. Thin-layer cells are often equipped with disc electrodes over which the analyte is directed in a thin layer of liquid. The performance of thin-layer electrochemical cells in terms of conversion efficiency depends on the contact time between the analyte and the electrode surface, which in turn is determined by the time needed for the analyte to diffuse to the surface in relation to its residence time in the electrochemical cell. This ratio can be significantly shifted in favor of electrochemical conversion by reducing the cell dimensions to microfluidic length scales. Employing microfabrication technologies based on photolithography enables shorter diffusion distances, reduced cell volumes, and rapid sample processing.¹⁷ Design aspects and various applications of microfluidic electrochemical cells coupled to MS have been described previously.¹⁸ On the basis of these principles, microfluidic electrochemical cells with an integrated platinum three-electrode system have been developed in our group to study drug metabolites in EC-MS experiments.^{19–21} While this kind of cells may also be exploited for peptide bond cleavage, platinum electrodes are not suitable for applications involving peptides and proteins due to adsorption at the surface.²²

While pure synthetic diamond is an insulator, it can be made conductive by appropriate doping.²³ Research on conductive diamond for electrochemical measurements started in the 1980s with ion implanted electrodes,²⁴ while Swain et al. extensively characterized the electrochemical properties of as-grown (untreated) polycrystalline BDD.²⁵ BDD exhibits various striking physical, chemical, and electrical properties, making it an attractive material for a variety of electrochemical applications. These include its mechanical stability, optical transparency, high chemical inertness, low double layer capacitance, low background currents and large overpotentials

for hydrogen and oxygen evolution.²⁶ The electrochemical properties of BDD electrodes can be further tailored to specific needs through a myriad of different surface modifications. These, along with BDD synthesis and characterization techniques, have been extensively reviewed.^{27–30}

In our current research, we integrated BDD electrodes into microfluidic electrochemical cells to combine the benefits of BDD with the advantages of a microfluidic electrochemical cell design for peptide bond cleavage. There have been a few reports on microstructuring BDD, such as laser micro-machining,³¹ lift-off,³² or dry etching techniques.^{33–36} Using inductively coupled plasma etching with O₂/Ar, Forsberg et al. fabricated BDD microband electrodes for electrochemical detection in poly(dimethylsiloxane) (PDMS) microchannels,³⁷ demonstrating the superior robustness and stability of BDD compared to gold electrodes. However, the PDMS channels could not be reused, possibly due to problems of analyte adsorption at PDMS, preventing repeated, sensitive electrochemical measurements.

Here we report for the first time on the design and fabrication of a robust and reusable glass-based microfluidic electrochemical cell with integrated BDD electrodes. This cell was used for peptide bond cleavage at the C-terminal side of tyrosine and tryptophan in the tripeptides leucine-tyrosine-leucine (LYL) and leucine-tryptophan-leucine (LWL). By coupling the device online to a high-resolution mass spectrometer, the relation between electrode potential and peptide bond cleavage was studied using a “mass voltammogram”. We further show that hydroxyl radicals ($\bullet$ OH) can be generated on BDD at elevated potentials, providing an alternative oxidation mechanism. This became evident from the aromatic hydroxylation of phenylalanine in the para-position to yield tyrosine in the tripeptide leucine-phenylalanine-leucine (LFL) with subsequent peptide bond cleavage. Applicability of the microfluidic cell to larger peptides and proteins is shown by specific cleavage of peptide bonds at the C-terminal side of tyrosine and tryptophan in ACTH 1-10, bovine insulin and lysozyme from chicken egg white, demonstrating the possibility of developing this into a novel device for protein analysis and proteomics research.

■ EXPERIMENTAL SECTION

Microfluidic Electrochemical Cell Fabrication. The electrochemical cell is constructed from two wafers: the first is a BDD-on-insulator wafer containing electrodes and microchannels, the second is a borosilicate glass wafer that contains access holes for electrical and fluidic connectivity and additional microfluidic structures. In the latter, 5 μ m deep structures were etched in a deep reactive ion etching process followed by powder blasting of access holes. To prepare the BDD-on-insulator wafer, 300 nm SiO₂ and 75 nm Si₃N₄ were grown on a p-type silicon wafer. Following this, a 300 nm thick diamond layer with a 10 000 ppm boron doping was grown in a process performed by Neocoat SA (La Chaux-de-Fonds, Switzerland). Here, diamond nuclei were seeded at high density, after which the BDD layer was grown in a hot-filament chemical vapor deposition process using CH₄ and trimethyl-boron in H₂. The BDD working and counter electrodes were structured using an O₂ reactive ion etching (RIE) process. Next, contact pads and reference electrodes were made from sputtered platinum (120 nm) on a tantalum (10 nm) adhesion layer in a lift-off process. Channel structures were patterned over the electrodes in a 5 μ m thick layer of SU-8, upon which

Figure 1. Schematic representation of the microfluidic electrochemical cell. (A) Photo of an assembled device. (B) Exploded view of the cell, showing the different layers of structures. (C) Layers of structures containing a BDD working electrode (WE) and counter electrode (CE) (I), a Pt pseudoreference electrode (pRE), and Pt contact pads (II). Microchannels were prepared in 5 μm thick SU-8 using photolithography (III) and additional 5 μm deep microfluidic structures and access holes were etched and powder blasted, respectively, in borosilicate glass (IV). (D) Schematic diagram of fluidic structures, indicating the channels located on top of the WE and CE. (E) Expanded view of part of the WE and frit channel network.

the two wafers were immediately aligned and bonded together at elevated pressure and temperature in an anodic bond tool (EV-501, EVG, Austria). Finally, the bond strength was increased at 180 $^{\circ}\text{C}$ and 19 kg/cm^2 for 1 h using a hydraulic press (model 3889, Carver Inc.).

Chemicals. The tripeptides LWL and LYL were obtained from Research Plus Inc. (Barnegat). LFL was purchased from Bachem (Weil am Rhein, Germany). Potassium ferricyanide, potassium ferrocyanide, potassium nitrate (KNO_3), potassium dihydrogen phosphate, dipotassium hydrogen phosphate, 1,1'-ferrocenedimethanol, human adrenocorticotrophic hormone (ACTH) 1-10 (SYSMEHFRWG), chicken egg white lysozyme, insulin from bovine pancreas, iodoacetamide (IAM), dithiothreitol (DTT), ammonium bicarbonate (99.5%), and formic acid (98%) were obtained from Sigma-Aldrich (Steinheim, Germany). Acetonitrile (HPLC SupraGradient grade) was purchased from Biosolve (Valkenswaard, The Netherlands). Water was purified by a Millipore system (resistivity 18.2 $\text{M}\Omega\text{ cm}$, Millipore Corp., Billerica). See section S1 in the Supporting Information for sample preparation procedures.

Instrumentation and Measurements. Prior to use, the microfluidic electrochemical cell was flushed with electrolyte solution followed by cyclic voltammetry scans (-2 to 2 V, 100 mV/s) until reproducible CVs were obtained. Analyte solutions were introduced at a total flow rate of 2 $\mu\text{L}/\text{min}$ using a syringe pump (Nemesys, Cetoni, Korbussen, Germany) installed in a Lab-in-a-Suitcase.³⁸ Cyclic voltammetric and chronoamperometric measurements to characterize the microfluidic electrochemical cells were done using a potentiostat (SP 300, Bio-

Logic, Claix, France). UV-vis absorbance measurements were done in a 2.4 μL cell with an optical path length of 10 mm (LWCC-M-10, World Precision Instruments), a deuterium lamp as light source (DH-2000, Ocean Optics) and a UV-vis spectrometer (Maya 2000 Pro, Ocean Optics).

Mass voltammograms of LWL, LYL, and LFL were recorded online by ramping the cell potential linearly from 0 to 2500 mV at a scan rate of 2 mV/s using a portable potentiostat (SP 200, Bio-Logic, Claix, France). A metal union connected to electrical ground was placed between the chip outlet and electrospray needle to decouple the electrochemical cell from the high voltage ESI interface. For ACTH 1-10, insulin, and lysozyme, the potential was ramped from 0 to 2000 mV under otherwise equal conditions. The electrochemical oxidation and cleavage products were analyzed using an LTQ-Orbitrap XL mass spectrometer (Thermo Scientific, Bremen, Germany). The transit time of 1.5 min between product formation within the electrochemical cell and mass spectrometric detection was taken into account when the cell potential was synchronized with MS signal intensities.

Cleavage at constant potentials was done under conditions identical to the online EC-MS experiments using 1300 mV (LWL) or 2000 mV (LYL, insulin, and lysozyme). Cleavage of ACTH 1-10 was performed at two different constant potentials of 700 and 1100 mV. Oxidation of LFL by $\cdot\text{OH}$ radicals was shown to occur at a constant potential of 2000 mV. The reaction product mixtures were collected and diluted with water to 2.5 μM (LWL, LYL, LFL, ACTH 1-10, and insulin) and 1 μM (lysozyme) and analyzed by LC-MS/MS. The cleavage

yield was calculated from the peak area of the cleavage product (A_{cl}) and the total area of the peaks of uncleaved tyrosine and tryptophan oxidation products (A_{ox}):

$$\text{yield} = \left| \frac{A_{cl}}{A_{cl} + A_{ox}} \right| \times 100\% \quad (1)$$

LC–MS/MS analyses of cleavage product mixtures were performed on LC systems coupled to an LTQ–Orbitrap XL mass spectrometer. The tripeptides and ACTH 1–10 were separated using a Dionex Ultimate 3000 nano-LC system equipped with an Acclaim Pepmap column (150 mm $\times$ 75 μ m (length $\times$ i.d.), Thermo Scientific, Bremen, Germany) with a 40 min gradient of 2–50% acetonitrile in water/0.1% formic acid at a flow rate of 300 nL/min. Electrochemically cleaved insulin and reduced and alkylated lysozyme were separated using an HPLC system equipped with a Shim-pack XR-ODS column (50 mm $\times$ 2.0 mm (length $\times$ i.d.), Shimadzu, Kyoto, Japan) with a 30 min gradient of 2–60% acetonitrile in water/0.1% formic acid at a flow rate of 300 μ L/min. See section S1 in the Supporting Information for MS equipment settings and search engine parameters (PEAKS version 7.5, Bioinformatics Solutions Inc.) used to analyze LC–MS/MS data with the SwissProt (chicken) database.

RESULTS AND DISCUSSION

Microfluidic Electrochemical Cell Design. A photo and exploded view of the three-electrode microfluidic electrochemical cell are shown in Figure 1A,B, respectively. The device consists of four layers with microfabricated structures, which are constituted as shown in Figure 1C; (I) a BDD-on-insulator wafer with the working electrode (WE) and counter electrode (CE) structured in BDD, (II) the pseudoreference electrode (pRE) and electrical contact pads made from platinum, (III) microchannel structures in SU-8, and (IV) a borosilicate glass wafer with powder blasted access holes and etched microfluidic structures.

Following the inlet of the electrochemical cell, a T-junction directs the flow into two separate channels, one located above the WE and the other located above the CE (see Figure 1D). This ensures separation of the respective reaction products. The pRE is located in close proximity to the WE (300 μ m distance) just before the junction to minimize the electrolyte resistance between these two electrodes and prevent an unwanted Ohmic drop.

The conversion performance of a thin-layer type electrochemical cell can be related to a dimensionless number in analogy to the performance of a separation column, which is usually described by the number of equilibrium stages (theoretical plates). For thin-layer electrochemical cells, this plate number (N_{dl}) can be defined as a function of both the residence time of a molecule above the electrode (t_{res}) and the time it takes for this molecule to diffuse to reach the electrode surface (t_d).¹⁸ This number is therefore a function of cell geometry (channel height (h) and length of the channel in contact with the electrode (l)), the diffusion coefficient of the respective molecule (D), and the average flow velocity ($\bar{u}$). Alternatively, N_{dl} can be calculated using the liquid volume in contact with the electrode (V) and the volumetric flow rate (Q):

$$N_{dl} = \frac{t_{res}}{t_d} = \frac{2Dl}{\bar{u}h^2} = \frac{2DV}{Qh^2} \quad (2)$$

On the basis of these considerations related to mass transport, we designed a cell with shallow, long meandering channels having a width (w) of 200 μ m, $h = 5$ μ m, and $l = 24$ mm. The cell is typically operated using a 1 μ L/min flow rate over the WE and the volume above the WE is 24 nL. Using eq 2 with $D = 1.1 \times 10^{-10}$ m²/s for lysozyme,³⁹ we find that $N_{dl} = 13$, indicating that a high electrochemical conversion efficiency can be expected compared to most regular thin-layer flow cells, which have a $N_{dl} < 1$ for this protein.¹⁸ From a practical point of view, it is clear that the channel height is the most important length scale that determines the electrochemical cell's conversion performance, and therefore it is essential that this dimension can be accurately controlled in the microfabrication process.

To promote ionic conductance between the WE and CE and to generate a uniform current density over the electrodes, a network of narrow channels (frit channels) is located between the WE and CE channels. Previously, it has been shown that the use of frit channels can significantly enhance the electrochemical conversion efficiency of a microfluidic cell.²¹

The electrical resistance R_{el} of a single channel with geometric parameters l , w , and h and electrolyte conductivity κ (S/m) is given by

$$R_{el} = \frac{l}{\kappa wh} \quad (3)$$

To achieve sufficient conductance, the electrical resistance needs to be low and uniform throughout the network, while hydraulic resistance has to be sufficiently high to limit convection causing unequal flows above the CE and WE and mixing of reaction products. In addition, the frit channels have to be long enough to avoid mixing of WE and CE products by diffusion at the time scale of a typical experiment (30–60 min). In this design, the shortest path length between WE and CE via frit channels is 3.6 mm.

By calculating the diffusion distance in a one-dimensional concentration gradient (x_d):

$$x_d = \sqrt{2Dt_d} \quad (4)$$

It can be estimated that a protein such as lysozyme ($D = 1.1 \times 10^{-10}$ m²/s) will diffuse from one electrode to the other in ~ 16 h, which is considerably longer than the time scale of a typical electrochemical protein cleavage experiment.

An enlargement of part of the frit channel network is shown in Figure 1E. The ladder network consists of 34 narrow channels (10–26 μ m wide, 5 μ m deep) connecting the WE channel to a larger channel (200 μ m wide, 10 μ m deep) that, in turn, connects to the CE via a second ladder network of equal dimensions. Using the electrical resistance of a single frit channel (R_{frit}) and the spacing between adjacent frit channels (R_{cc}), the electrical resistance of a ladder (R_l) consisting of N frit channels is calculated as

$$R_l = \left(\sum_{i=0}^{N-1} \frac{1}{R_{frit} + iR_{cc}} \right)^{-1} \quad (5)$$

which converges to R_{frit}/N for $R_{cc} \ll R_{frit}$.

After adding the resistance of the interconnecting channel (R_{ic}), the total frit channel network resistance R_t can be obtained from K identical branches in parallel with the channel connecting the WE to the CE at the junction (R_j):

$$R_t = \left(\frac{K}{2R_l + R_{ic}} + \frac{1}{R_j} \right)^{-1} \quad (6)$$

With these equations and the conductivity of 0.1 M KNO₃ as electrolyte (1.08 S/m), the total resistance between WE and CE for the chip design shown in Figure 1, containing 7 frit channel branches, is calculated to be 192 kΩ. This compares favorably to the first microfluidic electrochemical cell that was equipped with a frit channel network, which had a WE-CE resistance of 530 kΩ in the same electrolyte solution.²¹ See section S2 in the Supporting Information for considerations on the hydraulic resistance of this frit channel network and the use of hydraulic resistors close to the chip outlets.

BDD Material and Microfluidic Electrochemical Cell Characterization. The potential window of BDD electrodes was determined off-chip with a 0.1 M KNO₃/10 mM phosphate buffer (pH 7.4) solution. A microstructured BDD WE (4.8 mm²), using only the bottom part of a chip without the glass top layer (see Figure 1B,C) was compared to a platinum WE (2.5 mm²) in a macroscopic (regular) electrochemical cell having a platinum CE and a commercially available KCl saturated Ag/AgCl RE. Cyclic voltammograms (CVs) recorded at 100 mV/s with both WEs (Figure 2A) show that the BDD

Figure 2. (A) Determination of the potential window of a microstructured BDD WE compared to a platinum WE in a regular electrochemical cell setup containing 0.1 M KNO₃/10 mM phosphate buffer solution (pH 7.4). CVs were recorded at a scan rate of 100 mV/s using a saturated Ag/AgCl RE and a platinum CE. (B) Optical absorbance measurements of ferricyanide using 0.45 mM/0.45 mM ferri-/ferrocyanide in 0.1 M KNO₃/10 mM phosphate buffer solution (pH 7.4), which was introduced at a flow rate of 2 μL/min.

electrode has a mainly featureless CV over a potential range of −2 to 2.2 V, providing a potential window of 4.2 V, while this is only 2.6 V for platinum due to the onset of water electrolysis. The small anodic current peak in the CV of the BDD electrode just before the onset of oxygen evolution has been reported to originate from the oxidation of nondiamond carbon impurities at the surface.⁴⁰ To take maximum advantage of the

electrochemical properties of this material, both WE and CE of the microfluidic cell were made from BDD to minimize the extent of gas bubble formation in the microchannels at elevated potentials.

Electrochemical conversion efficiency was characterized by UV–vis spectroscopy using 0.45 mM/0.45 mM ferri-/ferrocyanide in a 0.1 M KNO₃/10 mM phosphate buffer (pH 7.4) solution introduced at a flow rate of 2 μL/min. Ferricyanide absorbs at 418 nm, allowing calculation of the conversion efficiency (η) from the absorbance peak heights during oxidation and reduction (A_{ox} and A_{red} , respectively) with respect to the initial absorbance (A_0):

$$\eta_{ox/red} = \left| \frac{A_{ox/red} - A_0}{A_0} \right| \times 100\% \quad (7)$$

Figure 2B shows the absorbance measurements at 418 nm as a function of time. After absorbance has stabilized at the initial value, 1 V is applied to the WE for 5 min. Subsequently, the potential is switched back to open circuit potential (E_{ocp}) for 10 min, after which −1 V is applied for 5 min. For this redox couple, an oxidation efficiency of 97% and a reduction efficiency of 95% was calculated.

To be able to relate the protein cleavage potentials in the microfluidic electrochemical cell to a KCl saturated Ag/AgCl reference electrode, the Pt pRE was calibrated using 1,1'-ferrocenedimethanol. The potential of the platinum pRE was determined to be 225 mV vs Ag/AgCl (KCl saturated) in 89/10/1 (v/v/v) water/acetonitrile/formic acid (pH 2.0) and 196 mV vs Ag/AgCl (KCl saturated) in 85/10/5 (v/v/v) water/acetonitrile/formic acid (pH 1.5). See section S3 in the Supporting Information for the related measurement data.

Electrochemical Cleavage of Tripeptides. Two different tripeptides (LWL and LYL) were employed to study the specific electrochemical cleavage of peptide bonds C-terminal to tyrosine and tryptophan in the microfluidic electrochemical cell. First, electrochemical oxidation and cleavage products were generated from the peptides LWL and LYL using a linear potential sweep in an online EC-MS experiment. The measured mass voltammograms allowed determination of the potential range over which cleavage occurred. Electrochemical oxidation and cleavage mechanisms, previously published by Roeser et al.,¹⁴ are shown in Scheme S4.

A decrease of the LWL signal in Figure 3A indicated the onset of electrochemical oxidation at ~800 mV. Oxidation efficiency increased further with increasing WE potential. Signal intensity for the cleavage product (LW+14) reached a maximum at 1300 mV, followed by a decrease at higher potentials (Figure 3B) likely due to the formation of other oxidation products. LC-MS analysis of the reaction products of LWL generated at 1300 mV revealed an oxidation yield of 95%, determined from a decrease in LWL signal intensity and a cleavage yield of 50% (see LC-MS chromatograms in Figure S5 and eq 1).

Electrochemical oxidation of LYL started at ~450 mV (Figure 3C). However, compared to LWL, oxidation yield was lower and spread over a potential range from 500 to 1750 mV. Signal intensity for LYL decreased a second time at 1750 mV, which may be attributed to further oxidation by hydroxyl radicals (see also the results obtained with LFL, Figure 3E,F). Formation of the cleavage product LY-2 started at 750 mV and increased until 1250 mV after which signal intensity remained rather stable up to the upper limit of the voltammogram at

Figure 3. Electrochemical cleavage of LWL, LYL, and LFL. Online EC-MS voltammograms of LWL, LYL, and LFL ($10\ \mu\text{M}$ in 85/10/5 (v/v/v) water/acetonitrile/formic acid) were recorded by ramping the potential from 0 to 2500 mV at a scan rate of 2 mV/s. Traces were extracted and plotted versus cell potential for (A) LWL (m/z 431.27), (B) LW+14 (m/z 332.16), (C) LYL (m/z 408.25), (D) LY-2 (m/z 293.15), (E) LFL (m/z 392.26), and (F) LFL cleavage product LY-2 (m/z 293.15).

2500 mV (Figure 3D). LC-MS analysis of the reaction products of LYL generated at 2000 mV revealed an oxidation yield of 100% and a cleavage yield of 30% (see LC-MS chromatograms in Figure S6 and eq 1).

LFL was used to monitor the formation of hydroxyl radicals at the BDD electrode. Previously, it was shown that LFL can be cleaved after conversion to LYL through hydroxylation at the para-position of the phenyl group.¹⁶ A slight decrease in signal intensity of LFL at 1750–2500 mV indicated that it was converted (Figure 3E). As proof of aromatic hydroxylation, the cleavage product LY-2 appeared at 1850 mV, and its abundance increased until the upper limit of the potential range of 2500 mV (Figure 3F). LC-MS analysis of the reaction products of LFL generated at 2000 mV showed an oxidation yield of $\sim 8\%$ and a yield of the subsequent cleavage of LYL of $\sim 30\%$ (see LC-MS chromatograms in Figure S7 and eq 1).

Electrochemical Cleavage of ACTH 1-10. To see whether the microfluidic electrochemical cell could also be used to cleave peptide bonds in larger peptides, ACTH 1-10 (SYSMEHFRWG), which has one tyrosine and one tryptophan, was studied using online EC-MS. Mass voltammograms show that cleavage of the peptide bond at the C-terminal side of tryptophan occurred first, starting at a potential of 600 mV and reaching maximum signal intensity at 730 mV (see Figure 4). Cleavage at the C-terminal side of tyrosine started at 730 mV and reached maximum signal intensity between 1000 and 1250 mV. These results show that selectivity in peptide bond cleavage may be achieved by controlling the applied potential and notably by addressing tryptophan alone or tryptophan and tyrosine together. Cleavage products generated at 700 mV and

Figure 4. Electrochemical cleavage of ACTH 1-10 (SYSMEHFRWG), which has one tyrosine and one tryptophan. Online mass voltammograms of ACTH 1-10 ($10\ \mu\text{M}$ in 89/10/1 (v/v/v) water/acetonitrile/formic acid) were recorded by ramping the potential from 0 to 2000 mV with a scan rate of 2 mV/s. Traces were extracted and plotted versus cell potential for A, ACTH 1-10 (m/z 433.86, charge 3+); B, SYSMEHFRW+14 (m/z 419.51, charge 3+); and C, SMEHFRW+14 (m/z 336.14, charge 3+).

1100 mV were analyzed by LC-MS. The tryptophan cleavage product (SYSMEHFRW+14) was observed upon electrochemical cleavage at 700 mV, while both SYSMEHFRW+14 and the combined tryptophan and tyrosine cleavage product (SMEHFRW+14) were observed at 1100 mV (see Figure S8 for LC-MS chromatograms).

Electrochemical Cleavage of Insulin. Electrochemical cleavage of bovine insulin was studied to see whether the approach could be extended to a significantly more complex molecule. Insulin is composed of 51 amino acids, including 4 tyrosines (numbered 1 to 4 and indicated in red in Figure 5A),

Figure 5. Electrochemical cleavage of insulin, which contains four tyrosine residues (shown in red). Insulin ($10\ \mu\text{M}$ in 85/10/5 (v/v/v) water/acetonitrile/formic acid) was electrochemically cleaved at 2000 mV. (A) Extracted ion chromatogram of insulin (m/z 1146.93, charge 5+) and (B) combined extracted ion chromatograms of the cleavage products TPKA (m/z 208.63, charge 2+), QLENY-2 (m/z 664.29, charge 1+), and A(1-14) + B(1-16) (m/z 1099.16, charge 3+).

which are assembled in 2 chains (A and B) that are linked by 2 disulfide bonds, while chain A has an additional internal disulfide bond. Mass voltammograms recorded using online EC-MS analysis (Figure S9) show that cleavage occurs over the potential range from 1200 to 2000 mV. Three cleavage products were detected: the C-terminal peptide formed upon cleavage at site 4 (TPKA), the peptide formed upon cleavage at sites 1 and 2 (QLENY-2), and the N-terminal parts of the A

Table 1. Electrochemical cleavage of chicken egg white lysozyme (reduced and alkylated with iodoacetamide): chicken egg white lysozyme contains 3 tyrosine and 6 tryptophan residues which are numbered as cleavage sites 1 to 9; lysozyme (2 μ M in 85/10/5 (v/v/v) water/acetonitrile/formic acid) was electrochemically cleaved at 1000 and 2000 mV

Full sequence of chicken egg white lysozyme (reduced and alkylated with IAM)				
KVFGRC ^a ELAA AMKRHGLDNY ¹ RGY ² SLGNW ³ VC ^a AAKFESNFNT QATNRNTDGS				
TDY ⁴ GILQINS RW ⁵ W ⁶ C ^a NDGRT GSRNLC ^a NIPC ^a SALLSSDITA SVNCA ^a AKKIVS				
DGNGMNAW ⁷ VA W ⁸ RNRC ^a KGTDV QAW ⁹ IRGC ^a RL				
Detected cleavage products	Cleavage site	Validation ^b	Monoisotopic mass (Da)	Cleavage potential ^c (mV)
SLGNW ³ +14	2, 3	M	589.2567	2000
KVFGRC ^a ELAAAMKRHGLDNY ¹ RGY ² SLGNW ³ +14	3	P	3283.5920	1000
VC ^a AAKFESNFNTQATNRNTDGS ⁴ TDY ⁴ -2	3, 4	P	2808.2042	2000
VC ^a AAKFESNFNTQATNRNTDGS ⁴ TDY ⁴ GILQINSRW ⁵ +14	3, 5	P	3891.8066	2000
GILQINSRW ⁵ +14	4, 5	P	1098.5774	2000
VAW ⁸ +14	7, 8	M	388.1820	2000
RNRC ^a KGTDVQAW ⁹ +14	8, 9	(P)	1503.7005	2000
IRGC ^a RL	9	P	773.4327	2000

^aCysteine(C) was alkylated with iodoacetamide after reduction of disulfide bonds with dithiothreitol. ^bP, validation by database search algorithm PEAKS; (P), validation by database search algorithm PEAKS with a low score; M, validation manually by MS/MS spectra. ^cCleavage potential: the potential at which the highest intensity of cleavage product signals was observed.

and B chains released upon cleavage at sites 1 and 3, which are linked together by a disulfide bond (A(1-14) + B(1-16)). The cleavage products generated at 2000 mV were analyzed and identified using LC–MS (see Figure 5). These results show that peptide bonds at all 4 tyrosines in insulin were cleaved at the BDD electrode of the microfluidic electrochemical cell. This is in accordance with the earlier work of Permentier and Bruins reporting the electrochemical cleavage of insulin in a flow-through cell equipped with a porous graphite electrode.¹⁵

Electrochemical Cleavage of Lysozyme. To investigate whether electrochemically mediated cleavage is applicable to a protein, lysozyme was studied, containing 129 amino acids with 3 tyrosines and 6 tryptophans, and 4 internal disulfide bridges. To facilitate cleavage, disulfide bonds were reduced and free SH groups alkylated with iodoacetamide prior to electrochemical oxidation.¹⁵

Mass voltammograms of lysozyme recorded using online EC–MS (Figure S10) indicated that two distinct potential regions exist, in which the MS signal intensity for lysozyme decreased first by 60% followed by a further decrease toward zero at higher potentials. It appeared that electrochemical cleavage at site 3 (a tryptophan, see Table 1 for numbering of cleavage sites) occurred between 800 and 1000 mV, resulting in the peptide KVFGRC(+57)ELAA-AMKRHGLDNY-RGYSLGNW+14, and that cleavage at site 9 started at 1900 mV, resulting in the peptide IRGCRL released from the C-terminus of the protein.

This potential-dependence indicates again that some selectivity can be achieved, as already observed for ACTH 1-10. Table 1 presents an overview of the eight identified cleavage products of lysozyme that were generated after peptide bond cleavage at 7 out of 9 possible sites (2 out of 3 tyrosines and 5 out of 6 tryptophans) at a potential of 1000 mV and 2000 mV. See Figure S11 for LC–MS chromatograms. The database search algorithm PEAKS was used for protein identification using a chicken protein sequence database (*Gallus gallus*

(chicken), SwissProt) resulting in a single significant match to lysozyme based on 5 identified peptides (peptides marked with validation P in Table 1). Manual inspection allowed the identification of 2 additional peptides (marked with M in Table 1), which were filtered out by PEAKS because of their short length. These results indicate that the microfluidic electrochemical cell has the potential to be used for protein and proteomics research.

The first report on the electrochemical cleavage of lysozyme described cleavage between two tryptophan residues (site 5) using a graphite rod anode.¹¹ In later work, Permentier and Bruins observed four peptides upon electrochemical digestion of lysozyme on a porous graphite electrode.¹⁵ Comparing our results to earlier data, we see that the same 5 out of 6 tryptophan and 2 out of 3 tyrosine residues were cleaved in both the work of Permentier and these experiments. However, a larger number of peptides of increased length were recovered from the microfluidic electrochemical cell, which is especially relevant for confident protein identification. This improvement is likely due to reduced adsorption at BDD working electrodes compared to porous graphite electrodes used in earlier studies and a high electrochemical conversion efficiency of our thin-layer cell in microfluidic format.

CONCLUSIONS

In this work, we demonstrate the development and use of a microfluidic electrochemical cell for protein identification studies. To this end, peptides and proteins were cleaved electrochemically in a microfluidic cell equipped with integrated BDD electrodes and a volume of 160 nL. Advantages of the cell design and the superior properties of BDD were exploited to be able to generate lysozyme cleavage products that allowed us to identify this protein by interrogating a sequence database as a proof of concept for future proteomics studies.

Tripeptides (LWL and LYL) were used to demonstrate specific electrochemical peptide bond cleavage at the C-terminal side of tyrosine and tryptophan, followed by cleavage of the decapeptide ACTH (1–10), which contains both a tyrosine and a tryptophan residue. Peptide bond cleavage was found to be potential-dependent with tryptophan being cleaved at a lower potential than tyrosine suggesting that some selectivity may be obtained by varying the potential. Next, bovine insulin was cleaved at all 4 tyrosines, and finally chicken egg white lysozyme was successfully identified in the UniProt_SwissProt database based on five electrochemically generated peptides. For each compound, mass voltammograms recorded using an online EC-MS setup allowed rapid screening for cleavage potentials using small amounts of sample (33–42 μL), after which electrochemically generated peptides were analyzed in more detail using LC-MS/MS.

Further improvements will be needed to increase cleavage yield. The possibility of a chemical labeling approach based on the reactive spirolactone at the C-terminus of the cleaved peptides to introduce affinity tags for enrichment opens further possibilities even in the absence of complete electrochemical peptide bond cleavage. The observed dependence of peptide bond cleavage on the applied potential opens further possibilities to achieve some selectivity. In this context it is interesting to note that, while cleavage at sites 3 and 9 in lysozyme occurred C-terminal to tryptophan, the potentials required for cleavage were different. While the potential providing the highest cleavage yield at site 3 was similar to that observed for ACTH 1–10 (700–800 mV), cleavage at site 9 required more than 1900 mV. This indicates that other parameters, such as sequence context, may contribute to defining the cleavage potential in addition to whether a tryptophan or tyrosine peptide bond needs to be cleaved. Electrochemical protein cleavage in a microfluidic format could thus become an attractive, fully instrumental approach for protein digestion in proteomics research applications and the analysis of biopharmaceuticals.

■ ASSOCIATED CONTENT

■ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.6b02413.

Sample preparation, data acquisition, and database searching; hydraulic resistance of the frit channel network; calibration pseudo-reference electrode against Ag/AgCl; electrochemical oxidation and cleavage mechanisms of LWL and LYL; LC-MS extracted ion chromatograms of oxidized and cleaved LWL and LYL, oxidized and cleaved LFL, and ACTH and lysozyme cleavage products; and mass voltammograms of insulin and lysozyme cleavage products (PDF)

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

- (1) Sobott, F.; Robinson, C. V. *Curr. Opin. Struct. Biol.* **2002**, *12* (6), 729–734.
- (2) Trauger, S. A.; Webb, W.; Siuzdak, G. *Spectroscopy* **2002**, *16*, 15–28.
- (3) Wysocki, V. H.; Resing, K. A.; Zhang, Q.; Cheng, G. *Methods* **2005**, *35*, 211–222.
- (4) Henzel, W. J.; Watanabe, C.; Stults, J. T. *J. Am. Soc. Mass Spectrom.* **2003**, *14* (9), 931–942.
- (5) Cottrell, J. S. *J. Proteomics* **2011**, *74* (10), 1842–1851.
- (6) Ma, B.; Johnson, R. *Mol. Cell. Proteomics* **2012**, *11* (2), O111.014902.
- (7) Lam, H.; Deutsch, E. W.; Eddes, J. S.; Eng, J. K.; King, N.; Stein, S. E.; Aebersold, R. *Proteomics* **2007**, *7*, 655–667.
- (8) Lam, H.; Deutsch, E. W.; Eddes, J. S.; Eng, J. K.; Stein, S. E.; Aebersold, R. *Nat. Methods* **2008**, *5* (10), 873–875.
- (9) Maleknia, S. D.; Johnson, R. In *Amino Acids, Peptides and Proteins in Organic Chemistry*; Hughes, A. B., Ed.; Wiley-VCH: Weinheim, Germany, 2012; Vol. 5, pp 1–50.
- (10) Iwasaki, H.; Cohen, L. A.; Witkop, B. *J. Am. Chem. Soc.* **1963**, *85* (22), 3701–3702.
- (11) Walton, D. J.; Richards, P. G.; Heptinstall, J.; Coles, B. *Electrochim. Acta* **1997**, *42* (15), 2285–2294.
- (12) MacDonald, S. M.; Roscoe, S. G. *Electrochim. Acta* **1997**, *42* (8), 1189–1200.
- (13) Permentier, H. P.; Jurva, U.; Barroso, B.; Bruins, A. P. *Rapid Commun. Mass Spectrom.* **2003**, *17* (14), 1585–1592.
- (14) Roeser, J.; Permentier, H. P.; Bruins, A. P.; Bischoff, R. *Anal. Chem.* **2010**, *82* (18), 7556–7565.
- (15) Permentier, H. P.; Bruins, A. P. *J. Am. Soc. Mass Spectrom.* **2004**, *15* (12), 1707–1716.
- (16) Roeser, J.; Alting, N. F. A.; Permentier, H. P.; Bruins, A. P.; Bischoff, R. *Anal. Chem.* **2013**, *85*, 6626–6632.
- (17) van den Brink, F. T. G.; Bütter, L.; Odijk, M.; Olthuis, W.; Karst, U.; van den Berg, A. *Anal. Chem.* **2015**, *87* (3), 1527–1535.
- (18) van den Brink, F. T. G.; Olthuis, W.; van den Berg, A.; Odijk, M. *TrAC, Trends Anal. Chem.* **2015**, *70*, 40–49.
- (19) Odijk, M.; Baumann, A.; Lohmann, W.; van den Brink, F. T. G.; Olthuis, W.; Karst, U.; van den Berg, A. *Lab Chip* **2009**, *9* (12), 1687–1693.
- (20) Odijk, M.; Baumann, A.; Olthuis, W.; van den Berg, A.; Karst, U. *Biosens. Bioelectron.* **2010**, *26* (4), 1521–1527.
- (21) Odijk, M.; Olthuis, W.; van den Berg, A.; Qiao, L.; Girault, H. *Anal. Chem.* **2012**, *84* (21), 9176–9183.
- (22) Odijk, M. *Miniaturized electrochemical cells for applications in drug screening and protein cleavage*. Ph.D. Thesis, University of Twente, Enschede, The Netherlands, 2011.
- (23) Fujimori, N.; Imai, T.; Doi, A. *Vacuum* **1986**, *36*, 99–102.
- (24) Iwaki, M.; Sato, S.; Takahashi, K.; Sakairi, H. *Nucl. Instrum. Methods Phys. Res.* **1983**, *209–210* (Pt 2), 1129–1133.
- (25) Swain, G. M.; Ramesham, R. *Anal. Chem.* **1993**, *65* (4), 345–351.
- (26) Xu, J.; Granger, M. C.; Chen, Q.; Strojek, J. W.; Lister, T. E.; Swain, G. M. *Anal. Chem.* **1997**, *69* (19), 591A–597A.
- (27) Compton, R. G.; Foord, J. S.; Marken, F. *Electroanalysis* **2003**, *15* (17), 1349–1363.

- (28) Kraft, A. *Int. J. Electrochem. Sci.* **2007**, *2*, 355–385.
- (29) Luong, J. H. T.; Male, K. B.; Glennon, J. D. *Analyst* **2009**, *134*, 1965–1979.
- (30) Pecková, K.; Musilová, J.; Barek, J. *Crit. Rev. Anal. Chem.* **2009**, *39* (16), 148–172.
- (31) Joseph, M. B.; Bitziou, E.; Read, T. L.; Meng, L.; Palmer, N. L.; Mollart, T. P.; Newton, M. E.; MacPherson, J. V. *Anal. Chem.* **2014**, *86*, 5238–5244.
- (32) Soh, K. L.; Kang, W. P.; Davidson, J. L.; Wong, Y. M.; Cliffl, D. E.; Swain, G. M. *Diamond Relat. Mater.* **2008**, *17* (4–5), 900–905.
- (33) Dorsch, O.; Werner, M.; Obermeier, E. *Diamond Relat. Mater.* **1995**, *4* (c), 456–459.
- (34) Otterbach, R.; Hilleringmann, U. *Diamond Relat. Mater.* **2002**, *11*, 841–844.
- (35) Enlund, J.; Isberg, J.; Karlsson, M.; Nikolajeff, F.; Olsson, J.; Twitchen, D. J. *Carbon* **2005**, *43*, 1839–1842.
- (36) Watanabe, T.; Shibano, S.; Maeda, H.; Sugitani, A.; Katayama, M.; Matsumoto, Y.; Einaga, Y. *Electrochim. Acta* **2016**, *197*, 159–166.
- (37) Forsberg, P.; Jorge, E. O.; Nyholm, L.; Nikolajeff, F.; Karlsson, M. *Diamond Relat. Mater.* **2011**, *20* (8), 1121–1124.
- (38) Odijk, M.; De Boer, H.; Olthuis, W.; van den Berg, A. In *14th International Conference on Miniaturized Systems for Chemistry and Life Sciences, μ TAS 2010*, Groningen, The Netherlands, October 3–7, 2010; pp 399–401.
- (39) Venable, R. M.; Pastor, R. W. *Biopolymers* **1988**, *27*, 1001–1014.
- (40) Granger, M. C.; Witek, M.; Xu, J.; Wang, J.; Hupert, M.; Hanks, A.; Koppang, M. D.; Butler, J. E.; Lucazeau, G.; Mermoux, M.; Strojek, J. W.; Swain, G. M. *Anal. Chem.* **2000**, *72* (16), 3793–3804.