Specific Affinity Enrichment of Electrochemically Cleaved Peptides Based on Cu(II)-Mediated Spirolactone Tagging

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ABSTRACT: Specific digestion of proteins is an essential step for mass spectrometry-based proteomics, and the chemical labeling of the resulting peptides is often used for peptide enrichment or the introduction of desirable tags. Electrochemical oxidation yielding specific cleavage C-terminal to tyrosine (Tyr) and tryptophan (Trp) residues provides a potential alternative to enzymatic digestion and a possibility for further chemical labeling by introducing reactive spirolactone moieties. However, spirolactone-containing peptides suffer from low stability due to hydrolysis and intramolecular side reactions. We found that Cu(II) ions stabilize the spirolactone and prevent intramolecular side reactions during chemical labeling, allowing efficient chemical tagging with a reduced excess of labeling reagent without intramolecular side reactions. On the basis of this reaction, we developed an analytical procedure combining electrochemical digestion, Cu(II)-mediated spirolactone biotinylation, and enrichment by avidin affinity chromatography with mass spectrometry. The method was optimized with the tripeptide LWL and subsequently applied to chicken egg white lysozyme, in which one biotinylated electrochemically cleaved peptide was identified after affinity enrichment. This proof-of-principle shows that specific enrichment of electrochemically cleaved spirolactone-containing peptides can be used for protein identification and notably that inclusion of Cu(II) ions is essential for stabilizing spirolactones for subsequent biotinylation.

Mass spectrometry-based proteomics is a powerful and indispensable tool for the analysis of complex samples,1−4 and the specific digestion of proteins plays a key role in their identification and quantification.5−8 Enzymes with different specificities are the cornerstone reagents for the digestion of proteins at specific peptide bonds5−9 while chemical cleavage is sometimes used as an alternative when a different specificity is required.10−13 Specific cleavage of the peptide bond C-terminal to tyrosine (Tyr) and tryptophan (Trp) residues occurs after electrochemical oxidation of peptides and proteins, which makes electrochemistry (EC) a potential instrumental alternative to chemical and enzymatic peptide bond cleavage.14−20 However, EC oxidation yields complex mixtures due to the generation of noncleavage oxidation products in addition to cleaved peptides, which poses a problem for proteomics applications.

Selective enrichment of proteins and peptides via affinity-based isolation has increasingly contributed to MS-based proteomics, serving to reduce sample complexity prior to LC-MS analysis.21−24 An efficient enrichment strategy for the peptides of interest via specific labeling would significantly reduce sample complexity and increase the depth of proteomics analyses.21,25,26 Biotinylation is a widely used strategy to prepare peptides and other molecules for enrichment by avidin affinity chromatography.27−32 Electrochemical cleavage of the peptide bond of Tyr or Trp yields a spirolactone moiety at the newly formed C-terminus providing a handle for specific chemical labeling.17,20 In previous work, we used this unique spirolactone to introduce affinity tags but encountered problems with spirolactone stability and intramolecular rearrangement.20

A number of chemical agents and biocatalysts are known to catalyze reactions between activated esters and primary amines including Lewis bases (e.g., pyridine, 2-hydroxypryidine),28,29 Lewis acids (e.g., sodium cyanide and salts of zinc, nickel, iron, lanthanum, and zirconium),30−33 and enzymes (e.g., Candida antarctica lipase B).37−39 For example, La(III) trifluoromethanesulfonate was developed as catalyst for synthesizing a variety of amides directly from esters and amines under mild conditions.34 We therefore investigated a range of potential catalysts in view of their ability to reduce side reactions and to obtain efficient coupling with a reduced excess of the often
costly labeling reagents. Addition of Cu(II) ions proved to be highly efficient in preventing spirolactone hydrolysis as well as intramolecular diketopiperazine formation. On the basis of this improvement, we developed a specific affinity enrichment method combining electrochemical digestion, Cu(II)-mediated spirolactone biotinylation, and affinity chromatography with LC-MS to identify proteins after electrochemical digestion. The method was first optimized with the tripeptide LWL and subsequently applied to chicken egg lysozyme.

**EXPERIMENTAL SECTION**

**Materials and Methods.** Formic acid (HCOOH, FA, 98%), dimethyl sulfoxide (DMSO, anhydrous, 99.8%), acetic acid anhydride (99%), chicken egg white lysozyme, iodoacetamide (IAM), dithiothreitol (DTT), ammonium bicarbonate (99.5%), Cu(II) chloride dihydrate (99.9%), hexylamine (99%), sodium phosphate (96%), sodium chloride (99.0%), pyridine (99.8%), 2-hydroxypropyridine (97.0%), sodium cyanide (97.0%), Cu(I) chloride (99.99%), nickel(II) chloride hexahydrate (98%), zinc acetate dihydrate (99.99%), iron(II) perchlorate hydrate (98%), iron(III) perchlorate hydrate (crystalline), and D-biotin (analytical standard) were purchased from Sigma-Aldrich (Steinheim, Germany). LWL was obtained from Research Plus Inc. (Barnegat, NJ, USA). Amine-PEG₂-biotin and monomeric avidin agarose were obtained from Pierce Biotechnology (Rockford, USA). Acetonitrile (HPLC grade) was purchased from Biosolve ( Valkenswaard, The Netherlands). Ultrapure water was obtained from a Milli-Q Advantage A10 water purification system at a resistivity of 18.2 MΩ cm (Millipore Corporation, Billerica, MA, USA).

**Peptide and Protein Preparation.** LWL at a concentration of 1 mM was prepared in 89/10/1 (v/v/v) water/acetonitrile/formic acid as stock solution. A stock solution of reduced and alkylated lysozyme was prepared in 89/10/1 (v/v/v) water/acetonitrile/formic acid at a protein concentration of 100 µM. For reduction and alkylation, lysozyme (100 µM) was prepared in 100 mM ammonium bicarbonate buffer (pH 8). Two mM DTT in 100 mM ammonium bicarbonate buffer was added, and the mixture was incubated with shaking at 500 rpm at 60 °C for 30 min. IAM was added at a concentration of 20 mM after cooling and reacting at room temperature in a dark environment for 40 min. After alkylation, 8 mM DTT was added to quench the alkylation reaction for 30 min. Lysozyme precipitated upon reduction and alkylation. The reaction mixture was centrifuged at 13 000 rpm, and the supernatant was removed. Water/acetonitrile/formic acid 89/10/1 (v/v/v) was added to dissolve the precipitated lysozyme and prepare a 100 µM stock solution of reduced and alkylated lysozyme. LWL was diluted to a final concentration of 10 µM (LWL) and lysozyme to 5 µM prior to electrochemical cleavage. To prevent formylation or acid hydrolysis, the formic acid content of lysozyme was increased to 5% just before the electrochemistry experiments. 10–13

**Electrochemical Cleavage.** Electrochemical oxidation and cleavage was performed in a µ-PrepCell electrochemical cell (thin-layer cell, Antec, Zoeterwoude, NL) with a boron-doped diamond (BDD, 12 × 30 mm × 1 mm) working electrode (Antec), a titanium counter electrode, and a palladium reference electrode (Pd/H₂). A flow rate of 10 µL/min was employed to introduce analyte solutions via a syringe pump (KD Scientific Inc., Holliston, MA, USA). The electrochemical potentials were controlled with a ROXY potentiotstat (Antec) operating in Scan and DC mode. Cathodic pretreatment of BDD electrodes at a negative potential of −3000 mV was used to regenerate the electrode surface prior to all experiments by pumping 0.5 M nitric acid in water at a flow rate of 50 µL/min for 1 h. Prior to use, the cell was flushed with electrolyte solution at a potential of −2000 mV for 1 h.

The optimal cleavage potentials of peptides and proteins were first determined via online EC-MS experiments by ramping the cell potential from 0 to 3000 mV linearly at a scan rate of 10 mV/s. The detection of electrochemical cleavage products in online EC-MS was achieved in an API 365 triple quadrupole mass spectrometer (PE-Sciex, Concord, Ontario, Canada) with an EPI+ upgrade (Ionics, Bolton, Ontario, Canada) in positive ion mode. LWL and lysozyme were oxidized at 1000 and 2000 mV vs Pd/H₂, and the product mixtures (EC-LWL and EC-lysozyme) were collected for LC-MS analysis and further reactions.

**Effect of Cu(II) on Stability of Cleavage Products and Chemical Tagging.** Cu(II) chloride dehydrate at a concentration of 100 µM was prepared in 89/10/1 (v/v/v) water/acetonitrile/formic acid as stock solution. EC-LWL with Cu(II) was prepared by adding 4 µL of Cu(II) chloride dehydrate (100 µM) into 2 mL of solution and dried following the same procedure as described in our previous work. 25 EC-LWL without Cu(II) was dried following the same procedure as the control.

To study the effect of Cu(II) on the stability of the spirolactone in peptides, 2 mL of EC-LWL with or without Cu(II) was concentrated by evaporation under nitrogen (2 h) and dried in an Eppendorf Concentrator at 30 °C. EC-LWL was prepared at a concentration of 400 µM by dissolving the dried sample in 50 µL of DMSO/TEA (99.9/0.1) by pipetting for 30 s. Reactions were performed at 25 °C with shaking at 900 rpm in an Eppendorf Thermomixer. Ten µL of reaction product mixtures at time point 0 and 6 h was prepared at a concentration of 5 µM by dilution with 390 µL of 99/1 (v/v) water/formic acid, and 40 µL of EC-LWL was subjected to LC-MS analysis. LC-MS analyses were performed on an Ultimate plus system (Dionex-LC Packings, Amsterdam, The Netherlands) connected to an API 365 triple quadrupole mass spectrometer (PE-Sciex) with an EPI+ upgrade (Ionics). The separation of the reaction mixtures was achieved on a Vyde RP-C18 column (150 mm × 2.1 mm i.d., 5 µm particles, 300 Å pore size, Grace Vydac, Lokeren, Belgium) with a 35 min gradient of 2–50% acetonitrile in water/0.1% formic acid at a flow rate of 250 µL/min.

Chemical tagging with hexylamine was performed at a concentration of 500 µM by adding 20 µL of a mixture of DMSO, TEA, and hexylamine (99.65:0.1:0.25) to 1 mL of a dried EC-LWL mixture in the presence or absence of Cu(II), respectively, with a 50-fold molar excess of hexylamine. The reaction mixtures were incubated with shaking at 900 rpm at 25 °C for 6 h and analyzed by LC-MS. Biotinylation of EC-LWL was performed at a concentration of 500 µM for peptides in the presence or absence of Cu(II). Prior to use, 2 mg of amine-PEG₂-biotin was prepared in 1 mL of water and purified on an Oasis HLB extraction cartridge (1 cm³, 30 mg, Waters Corporation, Milford, Massachusetts, USA) by elution with 15 mL of 10% ACN. After evaporating under nitrogen and drying in an Eppendorf Concentrator 5301 (Eppendorf, Hamburg, Germany), amine-PEG₂-biotin was dissolved in 50 µL of DMSO/TEA (99.9/0.1). Twenty µL of amine-PEG₂-biotin in solution was added to the sample in the presence or absence of Cu(II) and shaken at 900 rpm at 25 °C for 16 h.
with a 200-fold molar excess of amine-PEG₂-biotin. Biotinylated EC-LWL (Bio-EC-LWL) was diluted to 5 μM by adding 99/1 (v/v) water/formic acid, and 40 μL of the reaction mixtures was analyzed by LC-MS. Liquid chromatography was performed on a Waters UPLC I-class system (Waters Corporation, Milford, USA) with a Waters Acquity Peptide BEH C18 column (100 mm × 2.1 mm i.d., 1.7 μm particles, 300 Å pore size, Waters Corporation, Milford, USA) at 400 μL/min using a linear gradient from 5% to 40% acetonitrile in water/0.1% formic acid in 25 min. For mass spectrometry, a Maxis plus quadrupole time-of-flight mass spectrometer (QTOF, Bruker, Bremen, Germany) in positive electrospray ionization mode was used.

Biotinylation of EC-Lysozyme with Amine-PEG₂-Biotin. For biotinylation of EC-lysozyme with amine-PEG₂-biotin, a reaction was performed in the presence of Cu(II). EC-lysozyme with Cu(II) was prepared by adding 4 μL of Cu(II) chloride dehydrate (100 μM) into 2 mL of EC-lysozyme (5 μM) and dried following the same procedure as described in our previous work.²⁰ Two mg of amine-PEG₂-biotin was purified, dried, and dissolved in 50 μL of DMSO/TEA (99.9/0.1) as described above. Twenty μL of amine-PEG₂-biotin in DMSO/TEA (99.9/0.1) was added to the dried EC-lysozyme followed by pipetting for 30 s, and the mixture was incubated for 16 h with shaking at 900 rpm at 25 °C.

Removal of Excess Biotin by SPE after Biotinylation. The biotinylation reaction mixture was diluted with 1 mL of water/formic acid (99.9/0.1) and loaded on a Strata C18-E cartridge (55 μm, 70 Å, Phenomenex, Utrecht, The Netherlands). Twenty mL of 10% ACN and 3 mL of 15% ACN in H₂O/FA (99.9/0.1) were used to remove the excess of amine-PEG₂-biotin (200-fold) followed by elution of the biotynilated peptide by adding 3 mL of 50% ACN to the cartridge. Elution fractions were collected and concentrated by evaporation under nitrogen (2 h, 15 μL) at 30 °C. Bio-EC-LWL and bio-EC-lysozyme were diluted with 100 mM phosphate-buffered saline (PBS, 100 mM sodium phosphate, 150 mM sodium chloride, pH 7) to a final volume of 500 μL prior to affinity enrichment.

Affinity Enrichment of Biotinylated Peptides. One mL of monomeric avidin agarose was packed and prepared in a disposable column according to the supplier’s instructions. The biotinylated electrochemically cleaved peptides were captured with 1 mL of monomeric avidin agarose in the column by incubation for 30 min at room temperature. The immobilized monomeric avidin agarose column was washed with 2 mL of PBS. Biotinylated peptides were eluted with 2 mL of d-biotin at a concentration of 2 mM in 100 mM PBS and concentrated by evaporation using nitrogen at 30 °C for 2 h. The elution fractions of bio-EC-LWL and bio-EC-lysozyme were prepared to a final volume of 5 and 2 mL, respectively, by adding 99/1 (v/v) water/formic acid prior to LC-MS/MS analysis.

Analysis by LC-MS/MS. LC-MS/MS analyses of the EC-cleaved peptide mixture, the biotinylation reaction mixture, and the affinity-enriched biotinylated peptides were performed on a UPLC I-Class system (Waters) coupled to a quadrupole time-of-flight mass spectrometer (Maxis plus, Bruker) as described above. MS scans from m/z 200 to 1750 were recorded at a resolution of 75 000. MS/MS spectra were recorded at a resolution of 17 500 with a normalized collision energy of 35 V in data-dependent mode. The top 10 highest intensity peaks were chosen for MS/MS.

Data Analysis and Database Searching. The database search engine PEAKS (version 8.0, Bioinformatics Solutions Inc.) was used to analyze LC-MS/MS data using the chicken UniProt protein sequence database (Gallus gallus, updated 06–12–2016, SwissProt reviewed entries only) containing 2601 proteins. The search parameters were as follows: Parent Mass Error Tolerance, 10.0 ppm; Fragment Mass Error Tolerance, 0.05 Da; Enzyme, EC (custom-defined, digestion after Y or W); Max Missed Cleavages, 5; Nonspecific Cleavage, one; Variable Modifications, Oxidation (on MFWHYC), +15.99, Carbamyl-dimethylation (C), +57.02; EC-Y-2 (custom-defined, on C-terminal Y), −2.02; EC-W+14 (custom-defined, on C-terminal W), +13.98; EC-Y+372.16 (custom-defined, on C-terminal Y), +372.16; EC-W+388.18 (custom-defined, on C-terminal W), +388.18; Maximum variable post-translational modifications per peptide, 5.

RESULTS AND DISCUSSION

Stabilization of Peptide-Spirolactones against Intramolecular Rearrangement in the Presence of Cu(II) Ions. Specific cleavage after Tyr and Trp in peptides and proteins upon electrochemical oxidation yields a complex mixture of peptides with a reactive spirolactone moiety at the C-terminus of the N-terminal fragment in addition to other peptide and protein modifications.¹⁸–³⁰ We previously developed an approach to label electrochemically cleaved peptides via spirolactone chemistry to introduce affinity tags for subsequent enrichment by immobilized avidin chromatography and characterization by mass spectrometry. In this approach, acetylation of the amino group at the N-terminus was essential to prevent intramolecular rearrangement to nonreactive diketopiperazines. However, this approach has limitations when it comes to the electrochemical cleavage of larger peptides or proteins, because of the unavoidable generation of neo-N-termini and the increasing difficulty to achieve complete acetylation of all amino groups. Another limitation of this approach is that a large molar excess (2000-fold) of the affinity tag amine-PEG₂-biotin was required for efficient tagging. To prevent this unwanted side reaction and to increase coupling efficiency at lower molar excess of affinity tags, an alternative strategy is required that does not rely on acetylation.

To investigate a range of catalysts that have been described to facilitate the reaction between an activated ester and a primary amine, we screened the nucleophilic catalysts pyridine, 2-hydroxyppyridine and NaCN, as well as the following transition metal ions: Ni(II), Cu(I), Cu(II), Zn(II), Fe(II), and Fe(III). To study the effect of the selected catalysts, we followed the stability of LW+4 in DMSO containing 0.1% TEA as well as the reaction of LW+4 with a 50-fold molar excess of hexylamine. The presence of 0.5% pyridine or 2-hydroxyppyridine in the reaction mixture had no effect on the chemical coupling yield and accelerated the undesirable intramolecular rearrangement (data not shown). NaCN and most of the transition metal ions had no effect on either coupling yield or the stability of the spirolactone-containing...
peptides with respect to intramolecular rearrangement. Out of all tested reagents, we found that only addition of 2 μM Cu(II) ions prevented the intramolecular rearrangement reaction and significantly increased coupling yields with hexylamine. Figure 1A shows that the spirolactone-containing peptide LW+14 rearranges to a pair of isomeric diketopiperazines (LW+14*) in the absence of Cu(II), as previously described,20 while this was prevented by the addition of Cu(II). Rearrangement of LW+14 proceeded to 75% completion within 6 h in the absence of Cu(II), while LW+14 was stable in the presence of Cu(II) even under basic conditions in DMSO containing 0.1% TEA (Figure 1B). The small amount of diketopiperazines observed in the chromatogram was formed during the electrochemical cleavage reaction prior to adding Cu(II) ions.

**Cu(II)-Mediated Spirolactone Chemical Tagging.** As shown above, Cu(II) stabilized the spirolactone-containing peptide and prevented rearrangement to diketopiperazines without having to resort to acetylation. Consequently, the chemical coupling of LW+14 to hexylamine was studied in the absence of Cu(II) (A) and in the presence of Cu(II) (B) after incubation for 6 h in DMSO/TEA (99.9/0.1) at room temperature. For clarity, the 6 h traces in both (A) and (B) were offset by 1 min on the x-axis and by 5 × 10^5 cps on the y-axis.

![Figure 1. Stability of the electrochemical cleavage product LW+14 in the absence of Cu(II) (A) and in the presence of Cu(II) (B) after incubation for 6 h in DMSO/TEA (99.9/0.1) at room temperature. For clarity, the 6 h traces in both (A) and (B) were offset by 1 min on the x-axis and by 5 × 10^5 cps on the y-axis.](image)

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Reaction of LW+14 with amine-PEG₂-biotin at a 200-fold molar excess was investigated next. Figure 3A,B shows that only trace amounts of biotinylated LW+14 (Biotin-LW) were observed in the absence of Cu(II) ions since LW+14 underwent intramolecular rearrangement as the main reaction. Addition of Cu(II) prevented this side reaction resulting in complete biotinylation of LW+14 with amine-PEG₂-biotin under otherwise identical conditions (Figure 3C,D). These results confirmed that addition of Cu(II) is critical for efficient chemical tagging of spirolactone-containing peptides.

**Affinity Enrichment of Biotinylated Peptides.** Since electrochemical peptide bond cleavage not only generates the spirolactone-containing peptides, we investigated whether Cu(II)-mediated biotinylation can be used to enrich cleavage products from more complex mixtures. To this end, we combined Cu(II)-mediated biotinylation with selective affinity enrichment on monomeric avidin beads as shown in Figure 4A. Figure 4B shows the conversion of a Trp-containing peptide from electrochemical cleavage to biotinylation of the C-terminus of the N-terminal fragment.

![Figure 2. Chemical labeling of LW+14 with a 50-fold molar excess of hexylamine in the absence (A and B) and in the presence (C and D) of Cu(II). Extracted ion chromatograms of the electrochemically cleaved tripeptide LWL (LW+14, m/z 332.150) (A) before reaction, and of the chemical labeling product LW-hexylamine (433.256) (B) after reaction in DMSO containing 0.1% TEA for 6 h at room temperature in the absence of Cu(II). In the same order, panels C and D show extracted ion chromatograms of LW+14 (C) and the chemical labeling products LW-hexylamine (D) after reaction under the same conditions in the presence of Cu(II). Peaks marked with * depict the ion of LWL containing one 13C atom, which has the same mass as LW-hexylamine.](image)

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We first tested this approach on the tripeptide LWL. LWL was electrochemically cleaved at 1100 mV in an electrochemical cell with a BDD working electrode, yielding a mixture containing LWL, uncleaved oxidation products (LWL+32), and the spirolactone-containing cleavage product LW+14 (Figure 5A). LW+14 was completely biotinylated to LW-amine-PEG₂-biotin (Biotin-LW) in DMSO containing 0.1% TEA in the presence of Cu(II) resulting in a mass increment of 374.21 Da (Figure 5B). Excess amine-PEG₂-biotin was removed by SPE on a C18 cartridge, and the biotinylated peptide was captured on monomeric avidin agarose (Figure S1). After washing, biotinylated peptides were eluted with 2 mL of biotin (2 mM) in PBS. Figure 5C shows that biotinylated LW+14 (Biotin-LW) was effectively enriched from the complex mixture.

**Affinity Enrichment of Biotinylated Spirolactone-Containing Peptides from EC-Cleaved Lysozyme.** To study whether Cu(II)-mediated biotinylation after electrochemical peptide bond cleavage can be used to enrich spirolactone-containing peptides from proteins, we applied the procedure to lysozyme (chicken egg), a protein of 14.6 kDa. Lysozyme was electrochemically cleaved at 2000 mV, and the complex peptide mixture was biotinylated, enriched, and subjected to nanoLC-MS/MS. Data were subjected to searching the chicken UniProt protein sequence database (Gallus gallus, updated 06–12–2016, SwissProt reviewed entries only) containing 2601 proteins. Modifications such as biotinylation (mass increment of 374.2062 Da at the C-termini of predicted Tyr and Trp cleavage sites (resulting in an EC-Y+372.16 and an EC-W+388.18) were taken into account. The biotinylated peptide S4ILQUALW62+388.18, which derives from the spirolactone-containing peptide S4ILQINSRW62+14, was identified as a unique sequence of lysozyme. The MS/MS spectrum of the doubly charged precursor ion (m/z 737.8960) shows the characteristic, abundant fragment cations such as 737.8960/113.0100, 737.8960/332.1501, and 737.8960/332.1501 together with...
Fragments 1 to 4, which are smaller fragments of the amine-PEG2-biotin tag providing a signature of a biotin-tagged compound (Figure 6). This result provides proof-of-principle that it is possible to identify a protein by combining electrochemical peptide bond cleavage followed by Cu(II)-mediated biotinylation and selective affinity enrichment on monomeric avidin agarose. This approach will be further optimized and evaluated on more complex protein mixtures in view of future proteomics applications.

**CONCLUSIONS**

Electrochemical cleavage of peptide bonds C-terminal to Trp and Tyr opens the possibility for chemical labeling and enrichment via reactive spiro lactone moieties at the C-terminus of the N-terminal cleavage products. However, side reactions such as hydrolysis and intramolecular rearrangement during sample handling and chemical labeling proved to be challenges that needed to be overcome before this approach could be considered a potential instrumental alternative to chemical and enzymatic peptide bond cleavage in MS-based proteomics. In this work, we show that addition of Cu(II) ions stabilizes the spiro lactone moiety toward intramolecular rearrangement to diketopiperazines and with respect to hydrolysis. This allowed efficient chemical tagging of spiro lactones in the presence of free N-terminal amino groups. On the basis of this finding, we developed a method combining electrochemical peptide bond cleavage, Cu(II)-mediated spiro lactone biotinylation, and affinity chromatography with LC-MS/MS. This approach was
optimized with the tripeptide LWL and applied to lysozyme from chicken egg. We identified a unique biotinylated peptide \((^{34}\text{GILQINSRW}^{62}+388.18)\) in an unbiased “proteomics-like” approach using the algorithm PEAKS to search the sequence database of chicken \((\text{Gallus gallus})\). Despite this proof-of-principle study, there is need for further improvements, since we observed that spirolactones may already hydrolyze or rearrange during electrochemical cleavage and further sample handling, especially when positioned C-terminal to Tyr. We will investigate in future experiments whether Cu(II) ions can be added prior to electrochemical cleavage to prevent these side reactions. This refinement of the method should increase the recovery of electrochemically cleaved peptides significantly and facilitate subsequent biotinylation. In summary, we describe an approach for enriching electrochemically cleaved peptides from a mixture of electrochemical cleavage products that may serve as starting material for LC-MS/MS-based protein identification.

**ASSOCIATED CONTENT**

* Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.7b01039.

Specific capture of biotinylated peptides from a mixture of electrochemically cleaved, biotinylated LWL; proposed structures of the MS/MS fragment ions that are related to the amine-PEG2 biotin tag (PDF)

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**Figure 5.** LC-MS analysis of the electrochemical cleavage products of LWL (EC-LWL) (A), the reaction mixture after biotinylation and solid-phase extraction (SPE) (B), and the biotinylated LWL+14 (Biotin-LWL) after enrichment on monomeric avidin agarose (C). (A) Combined extracted ion chromatograms of the electrochemical cleavage products of LWL. The asterisk * indicates unoxidized LWL \((m/z 431.26)\). The symbol # represents uncleaved isomeric oxidation products LWL+32 \((m/z 463.26)\) next to LWL+14 \((m/z 332.16)\). (B) LC-MS analysis of the biotinylation products of EC-LWL after SPE purification to remove excess amine-PEG2-biotin showing complete biotinylation of LWL+14 \((m/z 332.16)\) to LWL-amine-PEG2-biotin \((\text{Biotin-LWL, } m/z 706.37)\) (mass increment of 374.21 Da). The more hydrophobic, later-eluting isomer of LWL+32 was also removed during SPE. (C) LC-MS analysis of biotinylation products of EC-LWL after enrichment on monomeric avidin agarose.

**Figure 6.** MS/MS spectrum of the biotinylated peptide \((^{34}\text{GILQINSRW}^{62}+388.18)\) resulting from electrochemical cleavage of chicken egg lysozyme. The peptide was enriched by monomeric avidin agarose after Cu(II)-mediated biotinylation. The fragment at \(m/z\) 375.2062 comprises the entire amine-PEG2-biotin tag, whereas Fragments 1 to 4 are parts thereof (see the proposed structures and \(m/z\) values of the fragments in Figure S2). The \(y\) and \(b\) fragment ions match the amino acid sequence.
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