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Exploiting charge differences for the analysis of challenging post-translational modifications by capillary electrophoresis-mass spectrometry

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ABSTRACT

Reversed-phase high-performance liquid chromatography (RP-HPLC) in combination with mass spectrometry (MS) is typically employed for mapping modifications in proteins and peptides. Here we applied a low-flow capillary electrophoresis (CE) –electrospray ionization interface coupled to Orbitrap mass spectrometers to analyze challenging modifications such as asparagine deamidation, aspartate isomerization, arginine citrullination, and phosphopeptide isomers. We achieved excellent resolution of asparagine (Asn), aspartic acid (Asp) and isoaspartic acid (iso-Asp) containing peptides using a synthetic peptide mixture. The migration order in CE enabled a clear assignment of *in vitro* deamidation/isomerization sites in a protein standard mixture of intermediate complexity (48 proteins) as well as the determination of the *in vivo* deamidation rate of histone H1.0 directly in a crude nuclear protein fraction. Besides these well-known modifications citrullination, a post-translational modification which changes the positively charged guanidinium group of arginine to the uncharged ureido group of citrulline, was investigated. Applying CE-MS for fast and sensitive analyses of various post-translational modifications of intact and enzymatically digested histone H4, we were able to detect a variety of citrullinated proteoforms. MS/MS analysis with electron transfer dissociation (ETD) fragmentation identified the presence of deiminated Arg at position 3 and 17 of histone H4. Moreover, based on CE-MS, isobaric mono-phosphorylated peptides obtained in the course of a kinase activity study were separated and individual positional isomers quantified.

1. Introduction

Currently, the most frequently employed technique for the analysis of deamidation products is mass spectrometry [1–4]. MS identification of deamidated peptides is relatively straightforward as deamidation adds 0.984 Da to the mass of a molecule. Collision-induced dissociation (CID) MS/MS spectra can usually reveal the exact position of deamidated Asn even in the presence of several Asn residues in the peptide sequence. Analytical techniques for the identification and quantification of iso-Asp and the elucidation of degradation pathways are much more challenging primarily because Asp and iso-Asp have identical mass and formal charge. CID is inefficient for distinguishing Asp and iso-Asp due to low specificity and unreliability [5]. A recent advance is the application of electron-capture dissociation (ECD) and electron transfer dissociation (ETD) [6,7]. These fragmentation methods produce reporter ions (c + 57 and z-57), which are unique to iso-Asp [2,8–10]. However, for large scale iso-Asp analysis using shotgun proteomics strategies, many of the initial hits based on reporter ions have been found to be false positives. The difficulty is mainly due to the low peak intensities for both reporter ions. When iso-Asp, Asp and Asn co-elute, the intensities of the reporter ions are further reduced and are not sufficient for unambiguous assignment. Moreover, not all peptides are amenable for ETD fragmentation, such as short and low-charged peptide ions. Therefore, a reliable pre-separation is an important prerequisite for the analysis of deamidation products, as it adds complementary information before MS analysis. Typical reversed phase HPLC conditions may lead to erroneous assignment, because the separation of the deamidation products is often poor and the elution order depends on the peptide sequence as well as on the chromatographic conditions [11]. Different chromatographic strategies have been developed for the detection

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of iso-Asp in proteins. Hao et al. described the use of electrostatic repulsion-hydrophilic interaction chromatography (ERLIC) for selective separation of deamidated peptides from rat liver tissue [12]. They identified 302 unique N-deamidated peptides, of which 20 were identified *via* all three deamidation-related products and 70 of which were identified *via* two of them. It was shown, that ERLIC effectively separates the structural isomers, however, further optimization is needed before it can be used efficiently with complex samples. Iso-Asp formation in monoclonal antibodies was studied utilizing specific digestion of the antibody with IdeS protease followed by hydrophobic interaction chromatography (HIC) separation [13]. Breaking the intact antibody into two smaller domains afforded better chromatographic separation and quantitation of iso-Asp containing species, which elute earlier than the Asp-containing peptide. However, this method cannot be used for complex samples. For a structure-based prediction of Asp and iso-Asp sites in antibody variable regions, Sydow et al. used RP-HPLC applying a Polaris C18-Ether column [14]. Deamidated species could be separated from the corresponding unmodified peptide, however, a more detailed assignment was not shown.

A further analytical challenge is the identification of citrullinated peptides. Citrullination is a post-translational modification converting protein arginine residues in the non-standard amino acid citrulline [15]. The conversion adds 0.984 Da to the mass of the intact molecule, which is the same mass increase as observed during deamidation. At physiological pH, arginine has a positive charge due to the guanidinium group, whereas citrulline is neutral. The conversion is catalyzed by a family of calcium-binding enzymes, called peptidylarginine deiminases (PAD) and is thought to alter protein structure, function and distribution. Recently, citrullination has become an area of significant interest, because of its suspected role in various pathological conditions, such as rheumatoid arthritis, multiple sclerosis and Alzheimer's disease [16]. The separation of citrullinated peptides from the corresponding non-citrullinated peptides is crucial for unambiguous distinction by MS.

Capillary electrophoresis, which allows separations based on mass to charge ratio of molecules, can be successfully applied to study post-translationally modified peptides and proteins. The change of net charge introduced by deamidation and citrullination as well as the slight differences between the pK_as of Asp and iso-Asp makes CE particularly well suited for this kind of analysis [17–19]. With recent developments in interfacing CE to electrospray ionization (ESI)-MS, CE has shown its application in protein and peptide analyses with its superior sensitivity and separation efficiency [20–25]. A new separation and ionization technology called CESI, which integrates the high efficiency of CE with ESI, relies on a separation capillary with a porous tip acting as nanospray emitter [26]. It has been demonstrated that this interface is able to work at flow rates less than 10 nL/min, which reduces ion suppression and improves sensitivity [27–31]. This CE-MS coupling was evaluated successfully for use in peptide and protein analysis, and was shown to be able to identify the localization of posttranslational modifications on antibodies and histones in a highly sensitive manner [32–35].

As there are few reports on CE-MS regarding its ability for the analysis of deamidation products [36–38] and none of citrullinated peptides, we systematically investigated CESI-MS for separation and differentiation of Asp and iso-Asp products due to Asn deamidation: i, a synthetic peptide mixture consisting of Asn, Asp and iso-Asp containing decapeptides was used to examine the migration behavior on CE; ii, a protein standard mixture of medium complexity (48 proteins) was digested with trypsin under different conditions to study the *in vitro* deamidation products and the reliability of the CE-MS method; iii, our method was applied to a crude nuclear protein fraction in order to directly evaluate the *in vivo* deamidation rate of histone H1.0.

The CE-MS method was also able to characterize arginine citrullination both on intact histone H4 and on peptides obtained after protease cleavage. The capability of CE to separate deimination- and deamidation products impressively shows how slight charge differences can be exploited for a successful MS analysis of even multiply modified proteoforms. For this reason, CE-MS was used as complementary approach for the quantification of mono-phosphorylated isobaric peptides, still a very challenging task in the proteomics field, as side specific product ions are often weak or not even detectable [39,40]. If such positional isomers cannot be separated, an unambiguous assignment of site-specific phosphorylation effects is problematic, a quantification often impossible. In the course of a kinase activity study isobaric mono-phosphorylated peptides were identified as potential substrates and therefore, the CE-MS approach was evaluated for their identification and quantification. As a result, a successful separation and quantification of individual positional isomers could be demonstrated.

2. Materials and methods

2.1. Materials

Trypsin (mass spectrometry grade) was obtained from Promega (Mannheim, Germany); 1,4 dithiothreitol was purchased from Biomol (Hamburg, Germany); and iodoacetamide from GE Healthcare (Vienna, Austria). Deamidated and acetylated forms of the histone H1.0 peptide with a sequence TENSTSAPAA were synthesized by Sigma Genosys (Cambridge, UK). The Universal Proteomics Standard (UPS1) containing 48 human proteins (5 pmoles each), endoproteinases Arg-C and Glu-C (sequencing grade), and all other chemicals were purchased from Sigma-Aldrich (Vienna, Austria). Water was purified with a Millipore Milli-Q Academic water purification system (Vienna, Austria).

2.2. Sample preparation

Proteins present in the Universal Proteomics Standard (UPS1) were solubilized in 10 μL ammonium bicarbonate buffer (10 mM, pH 8.0), reduced with 2 μL dithiothreitol (50 mM) at 56 °C for 30 min, and alkylated with 2 μL iodoacetamide (200 mM) at room temperature for 20 min. Proteins were digested at 37 °C for 18 h or 70 h using trypsin at a ratio of 1:20 (w/w). Histone H1 proteins were extracted from a human placenta according to Lindner et al. [41], solubilized in 5 mM ammonium bicarbonate buffer (pH 8.0) and digested for 1 h at 37 °C using endoproteinase Arg-C at a ratio of 1:20. Core histones were extracted from blood cells and fractionated by reversed-phase HPLC according to Helliger et al. [42]. The histone H4 fraction was dissolved in 10 mM sodium phosphate buffer (pH 7.8) and digested for 8 h at 37 °C using endoproteinase Glu-C at a ratio of 1:10. Resulting peptides were desalted using PerfectPure C-18 pipet tips (Eppendorf, Austria). The growth of SILAC labeled yeast strains, protein extraction thereof, and subsequent sample preparation was performed as described elsewhere [29]. All samples were lyophilized, re-solubilized in 50 mM ammonium acetate buffer (pH 4.0), and stored at −20 °C until analysis.

2.3. Capillary electrophoresis – mass spectrometry

For CE-MS analysis a CESI 8000 (Sciex, Brea, CA) equipped with a bare or neutrally-coated fused-silica capillary with a porous tip acting as nanospray emitter (Sciex, Brea, CA) (total length: 90 cm, i.d.: 30 μm, o.d.: 150 μm) was coupled *via* an ESI module to a Thermo Scientific Q Exactive Plus or LTQ Orbitrap XL ETD (Bremen, Germany). The neutral coating manufactured by Sciex comprises two layers, a hydrophobic coating and a hydrophilic polyacrylamide coating. The lower hydrophobic coating protects siloxanes