International Journal of Mass Spectrometry 353 (2013) 84–92

Contents lists available at ScienceDirect

International Journal of Mass Spectrometry

journal homepage: www.elsevier.com/locate/ijms

Short communication

Integration of online digestion and electrolytic reduction with mass spectrometry for rapid disulfide-containing protein structural analysis

Qiuling Zheng[a], Hao Zhang[b,*], Hao Chen[a,**]

[a] Center for Intelligent Chemical Instrumentation, Department of Chemistry and Biochemistry, Ohio University, Athens, OH 45701, USA
[b] Department of Chemistry, Washington University, St. Louis, MO 63130, USA

ARTICLE INFO

Article history:
Received 22 March 2013
Received in revised form 6 April 2013
Accepted 6 April 2013
Available online 18 April 2013

Keywords:
Mass spectrometry
Electrochemistry
Disulfide bond mapping
Protein sequencing
Selective reduction

ABSTRACT

Bottom-up structural analysis of disulfide-bond containing proteins usually involves time-consuming offline enzymatic digestion, chemical reduction and thiol protection prior to mass spectrometric detection, which takes many hours. This paper presents an expedited bottom-up approach, employing desorption electrospray ionization-mass spectrometry (DESI-MS) coupled with online pepsin digestion and online electrochemical reduction of disulfide bonds. Peptides are generated in high digestion yield as its precursor protein in acidic aqueous solution flows through a pepsin column, which can undergo direct electrolysis. The electrolytic behaviors of peptides, as online monitored by DESI-MS, suggest the presence or absence of disulfide bonds in the peptides, and also provide information to relate disulfide bond-containing peptide precursors to their corresponding reduced products. Furthermore, selective electrolysis simply using different reduction potentials can be adopted to generate either partially or fully reduced peptides to assist disulfide bond mapping. In addition, it turns out that DESI is suitable for ionizing peptides in water without organic solvent additives (organic solvent additives would not be compatible with the use of pepsin column). The feasibility of this method was demonstrated using insulin, a protein carrying three pairs of disulfide-bonds as an example, in which all disulfide bond linkages and most of the protein sequence were successfully determined. Strikingly, this method shortens the sample digestion, reduction and MS detection from hours to less than 7 min, which could be of high value in high-throughput proteomics research.

1. Introduction

Redox-active disulfide bonds are one of the most common protein post-translational modifications (~19% proteins contain multiple disulfide bonds [1]), which provide reversible covalent cross-linkages in native proteins for maintaining protein three-dimensional structures and their biological activities [2,3]. However, the presence of disulfide linkages increases the complexity for protein structure determination by mass spectrometry (MS). The cleavage of disulfide bond is often essential for protein/peptide analysis as dissociation of a reduced protein/peptide ion can give rise to more structurally informative fragment ions than that of the intact counterpart [2,3]. The traditional protocol to break a disulfide bond is chemical reduction using an excess amount of reagents like dithiothreitol (DTT) or tris(2-carboxyethyl)phosphine (TCEP). However, the reduction usually takes one-half to several hours and the removal of the excess amount of reductant is time-consuming. In addition, the resulting protein/peptide thiols often need to be protected due to the possibility of being re-oxidized prior to MS analysis. Besides chemical reduction, other novel approaches include the cleavage of disulfide bonds via laser-based ionization [4–7], ultraviolet photodissociation [8–10], negative ion dissociation [11–14], electron-capture dissociation (ECD)[15], electron-transfer dissociation (ETD)[16–20], plasma-induced oxidation [21], or using new ion chemistry [22–28]. An alternative way for reducing disulfide bonds without involving chemical reductants is electrolytic reduction [29,30].

For structural analysis of disulfide bond-containing proteins, bottom-up approach involving digestion of proteins into peptides is often adopted as it is easier to analyze smaller peptides than intact proteins. However, the conventional enzymatic digestion method is slow and requires overnight incubation, which can be another time-consuming step. Protocols for fast protein digestion have been introduced, such as digestion using modified enzymes [31], microwave digestion [32–34], ultrasonic-assisted protein digestion [35–38] and proteolysis accelerated by infrared radiation [38–40]. Results have been shown that protein digestion can be finished in minutes without reducing efficiency and accuracy. Pepsin packed column is another alternative method to minimize digestion time, due to high concentration of enzyme attached to the column [41].

* Corresponding author. Tel.: +1 314 935 7486.
** Corresponding author. Tel.: +1 740 593 0719; fax: +1 740 597 3157.
E-mail addresses: zhanghao@wuchem.wustl.edu (H. Zhang), chenh2@ohio.edu (H. Chen).

1387-3806/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.ijms.2013.04.009

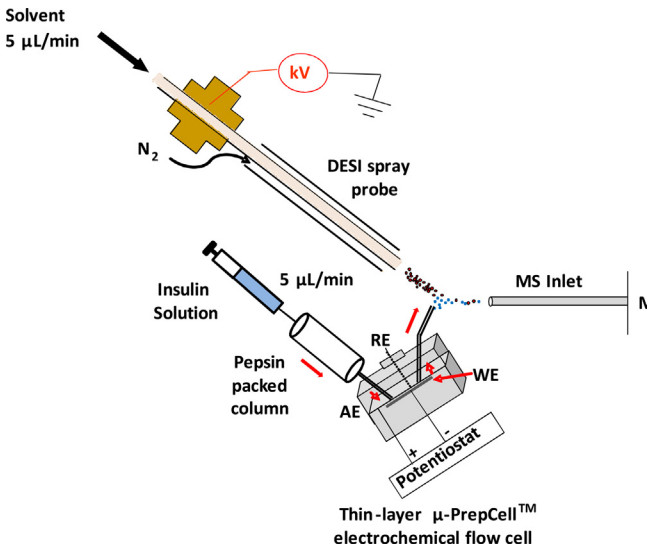
The online digestion using pepsin column has proved to be very useful for the analysis of proteins following hydrogen-deuterium exchange reactions [42], because backbone H/D exchange can be quenched at the acidic pH (typically pH 2.5) used for pepsin digestion. Recently, in our laboratory, online electrolytic reduction was combined with desorption electrospray ionization mass spectrometry (DESI-MS) for fast analysis of disulfide-bond containing proteins/peptides [43,44]. DESI-MS [45,46,47,48] is a recent advance in the field, which has been applied successfully to analysis of vast different analytes from pharmaceuticals to tissue imaging with little or no sample preparation. Our goal of integrating electrochemistry (EC) with DESI-MS (i.e., EC/DESI-MS method) is twofold. One is to use DESI-MS to study electrochemical reaction mechanisms in consideration of high specificity of MS detection; for instance, by online DESI-MS monitoring, the short-lived intermediate of chlorpromazine radical cation from chlorpromazine oxidation in an electrochemical cell was captured and the electrochemical nitroreduction mechanism was elucidated [49]. The other one is to find applications of the EC/DESI-MS method in proteomics. It has been shown that the EC/DESI-MS is useful for the structural analysis of disulfide bond containing proteins/peptides in either top-down [44] or bottom-up approach [43]. In the top-down approach employing ECD for ion dissociation, the number of fragment ions of lysozyme and β-lactoglobulin A increased to 3–13 fold after electrolytic reduction in comparison to those from the intact proteins [44]. In our previous bottom-up analysis approach [43], peptides and proteins were digested and then underwent online electrolysis and DESI-MS detection. Several useful findings were uncovered, including: (i) the disulfide-containing peptides in the digest mixture can be quickly identified, simply based on the abrupt decrease in their relative ion abundances after electrolysis and (ii) based on the mass relationship, precursor ions and their corresponding reduced product ions can be recognized [43]. However, in such a study, overnight trypsin digestion was a time-consuming step. The EC/DESI-MS method would be further benefited if online enzymatic digestion could be adopted.

In this study, we present an expedited method for disulfide bond-containing protein structural analysis, using online digestion and online electrolytic reduction combined with online MS analysis. In the experiment, by using a pepsin packed column as an immobilized enzyme reactor, fast protein digestion is achieved in high yield without overnight incubation. Insulin was selected as a model protein to demonstrate the feasibility of this new protocol. It turns out that protein can be quickly digested just as the aqueous solution is flowed through the pepsin column. The resulting peptides in the solution without organic solvent additives can be well ionized by DESI-MS. In addition, as reported before [43], based on the intensity changes before and after electro-reduction and their mass relationships, precursor ions and their corresponding reduced product ions were recognized. Furthermore, selective electrolysis was adopted to generate either partially or fully reduced peptides to assist disulfide linkage assignments. Results show that most of the protein sequence structure as well as all three disulfide bond locations of the examined protein were successfully determined using this new method. The method is very fast and it takes <7 min from sample injection to MS detection of peptide signal. This method is also green as no chemical reductant was used.

2. Materials and methods

2.1. Materials

Bovine pancreas insulin was purchased from Sigma–Aldrich (St. Louis, MO). Formic acid was purchased from Spectrum Chemical Mfg. Corp (Gardena, CA). HPLC-grade methanol was purchased from Fisher Scientific (Fair Lawn, NJ). The de-ionized water used for sample preparation was obtained using a Nanopure Diamond Barnstead purification system (Barnstead International, Dubuque, IA).



Scheme 1. Schematic showing the apparatus of the integrated online digestion, online electrolytic reduction and online MS analysis, for disulfide-bond containing protein structural analysis. WE: working electrode; AE: auxiliary electrode; and RE: reference electrode.

2.2. Methods

The home-built apparatus for online coupling a thin-layer electrochemical flow cell with a Thermo Finnigan LCQ DECA ion trap mass spectrometer (San Jose, CA) by liquid sample DESI [50] was used and described before (Scheme 1). A pepsin packed column was added between the sample injection syringe and the electrochemical cell. The sample syringe, pepsin packed column and thin-layer flow cell were connected using two short pieces of PEEK tubes (length 4.5 cm, i.d. 0.25 mm). 5 μM intact insulin (MW: 5733.5 Da) in 1% formic acid, was flowed through the pepsin packed column at a rate of 5 μL/min [41]. A thin-layer μ-PrepCell™ electrochemical flow cell equipped with a magic diamond (MD) electrode (12 mm × 30 mm, Antec BV, Netherlands) as the working electrode (WE) was employed and a Roxy™ potentiostat (Antec BV, Netherlands) was used to apply a reduction potential to the cell for triggering electrolytic reduction. The reduced species flowed out of the thin-layer cell via a piece of fused silica capillary (length 17 cm, i.d. 0.1 mm) and then underwent ionization by DESI. The spray solvent for DESI was methanol/water (1:1 by volume) containing 1% formic acid at the injection rate of 5 μL/min. A high voltage of +5 kV was applied to the DESI spray probe. Collision induced dissociation (CID) was carried out to provide ion structural information.

3. Results and discussion

Bovine pancreas insulin (MW 5733.5 Da, 51 amino acids, sequence is shown in Table 1) is known to have A and B chains linked by two inter-chain disulfide bonds, and the chain A of insulin has an additional intra-chain disulfide bond. Fig. 1a shows DESI-MS spectrum of the intact insulin in which +4, +5, and +6 of protein ions were observed at *m/z* 1434, 1147, and 957, respectively. Once the sample solution was flowed through the pepsin packed column at a flow rate of 5 μL/min, the protein was completely digested, as evidenced by the observation that all these protein peaks