Published in final edited form as:

Proteomics. 2013 November ; 13(22): 3245–3246. doi:10.1002/pmic.201300445.

Finding the Missing Link: Disulfide-containing proteins via a high-throughput proteomics approach

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Abstract

Top-down proteomics have recently started to gain attention as a novel method to provide insight into the structure of proteins in their native state, specifically the number and location of disulfide bridges. However, previous techniques still relied on complex and time-consuming protein purification and reduction reactions to yield useful information. In this issue of *Proteomics*, Zhao *et al.* (High throughput Screening of Disulfide-Containing Proteins in a Complex Mixture, *Proteomics* 2013) devise a clever and rapid method for high-throughput determination of disulfides in proteins via reduction by tris(2-carboxyethyl)phosphine. Their work provides the foundation necessary to undertake more complex experiments in biological samples.

Keywords

Disulfide; Glutathionylation; Mass spectrometry; Post-translational Modification; Redox Proteomics; Redox Modifications

As mass spectrometry (MS) enters the mainstream, novel methods are being devised to diversify the experimental questions that can be answered. Protein structure has usually been investigated by X-Ray crystallography and nuclear magnetic resonance, however, these techniques requires highly purified protein crystals that are not amenable to high-throughput screening. Fortunately, MS techniques have proved to be a complimentary approach to provide structural information about proteins. Indeed, precise determination of disulfide bonds between cysteines (Cys) is an essential first step to examine protein structure. Current MS methods to reveal disulfide bridges generally employ bottom-up techniques, with proteolytic digestion and extensive, difficult sample preparation such reduction and alkylation [1, 2]. These methods, while useful, do not allow for high-throughput analysis of complex samples and provide an incomplete image of disulfide bonds depending on sequence coverage of peptides recovered from digestion. More recently, top-down approaches have been used to provide a comprehensive analysis of disulfide bonds, albeit in purified protein samples [3].

In this issue [4], Zhao *et al.* expand on their previous work in the characterization of disulfide bonded proteins of human salivary α -amylase and adapt a relatively simple liquid chromatography (LC) technique with novel sample preparation to significantly enhance their method [3]. The aim of this study was to be able to develop a top-down LC/MS approach that allows for the rapid determination of disulfide bonds in complex protein mixture. This

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Conflict of Interest to Disclose:

None

important improvement is necessary in order to undertake high-throughput screenings of disulfide bridges in proteins in conditions where the reduction-oxidation (redox) balance of the cell is altered [2, 5]. Indeed, several pathologies display increased reactive oxygen species formation leading to modified Cys reactivity, such tissue following ischemia/reperfusion [6], Alzheimer's disease [7], oncogenesis [8] and muscular dystrophy [9]. Understanding the role of modified disulfide links in cellular degeneration might open new avenues for therapeutic intervention.

The authors use a clever approach, tris(2-carboxyethyl)phosphine (TCEP) in combination with low pH to reduce their protein mixture. This approach to reduction has the advantage of preventing disulfide scrambling and precludes alkylation of free Cys residues. TCEP is also compatible with LC/MS applications and does not require removal prior to running the samples. Thus, by comparing the molecular weights of a reduced and non-reduced protein, the number of the disulfide bonds can be inferred owing to an increase of two Dalton in mass per bridge. Interestingly, the authors use a lower resolution quadrupole time-of-flight (Q-TOF) MS, as opposed to a high resolution Fourier transform ion cyclotron resonance (FTICR) MS, usually employed in top-down proteomic, further highlighting the accessibility of the method. Their protein mixture contained bovine serum albumin (BSA), ubiquitin (Ubi), cytochrome C (Cyt C), β -lactoglobulin (BLG), lysozyme (Lyso), and ribonuclease B (RNase B) and was separated using traditional C18 reverse phase column, with all the proteins eluting in less than 25 minutes. Reduction of disulfide bridges altered retention time (RT), however, the changes in RT were not proportional to the numbers and cannot serve as a discriminating criteria for disulfide-containing proteins. As expected, the authors confirm that RNase B and Lyso have four disulfide bonds and BLG has two, whereas Ubi and Cyt C had none. More interesting, however, is the ability of Zhao *et al.* to rapidly determine the number of disulfide bonds in BSA, an astounding seventeen, in two sequential runs of the reduced and non-reduced form of the protein, despite poor mass resolution masking the isotopic distribution. Furthermore, confounding factors, such post-translational modifications (i.e. glycosylation), and protein isoforms did not impede the proper identification of disulfide bond number.

It remains to be seen how far this method can be pushed in terms of sample complexity. Whole cell lysates, or even sub-cellular fractions, present much more difficulty in terms of analysis than a simple mixture of six purified proteins. Systematic and unbiased comparison between the reduced and non-reduced sample runs will be needed to determine molecular weight differences, especially in light of varying RT. Additionally, the authors' choice of using a LC-Q-TOF-MS approach prevents prospective protein identification. Future studies using this method with MS/MS, and with higher resolution instruments, will reveal unknown disulfide bond containing proteins. This would also allow investigation of how post-translational modifications might affect disulfide formation, as major strength of top-down proteomics. One other potential application of the method is identifying quaternary structures between homodimers linked by disulfide bridges. These have recently come to light in redox sensitive signaling proteins, such as NF κ B [10] and Bak [11].

In summary, this novel method is easy to implement, provides crucial information about the number of disulfide bridges in a protein, and is likely to help future studies interrogating at disulfide bridges in cells and their regulation by the cytosolic redox state.

Acknowledgments

Dr. Sadayappan was supported by National Institutes of Health grants R01 HL105826 and K02 HL114749.

Abbreviations

LC	Liquid chromatography
MS	Mass spectrometry
Cys	Cysteines
TCEP	Tris(2-carboxyethyl)phosphine
Q-TOF	Quadrupole time-of-flight
FTICR	Fourier transform ion cyclotron resonance
BSA	Bovine serum albumin
Ubi	Ubiquitin
Cyt C	Cytochrome C
BLG	β -lactoglobulin
Lyso	Lysozyme
RNase B	Ribonuclease B
RT	Retention time
Redox	Reduction-Oxidation

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