Novel method for efficient reduction of disulfide bonds in peptides and proteins prior MS detection

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Introduction

Disulfide bonds are one of the most important post-translational modifications of proteins. They are stabilizing protein's 3-dimensional structure and are crucial for their biological function. Their presence can, however, hamper protein characterization by mass spectrometry. Proteins with disulfide bonds show more resistance to fragmentation and therefore need to be reduced prior MS analysis.

Off-line reduction is performed using highly concentrated chemical agent (*e.g.* dithiothreitol (DTT)) that needs to be removed prior LC/MS analysis. Alternatively, thiol - free reducing agents as TCEP (tris (2-carboxyethyl) phosphine) can be used. However, the sample preparation remains laborious and difficult to combine with on-line LC/MS.

Moreover, the possibility of on-line disulfide bond reduction can be beneficial for the determination of disulfide bond arrangements or top down proteomics strategy, which relies on fragmentation of intact proteins without enzymatic digestion.

Methods/Instrumentation

A preparative electrochemical cell (Antec) equipped with a Magic Diamond (MD) or a proprietary semi-precious metal working electrode was used for reduction of disulfide bonds. Typically, 1 - 3 µM solutions of the target compound (peptide, protein, etc.) in 1% formic acid /acetonitrile (90/10, v/v) were pumped through the electrochemical (EC) cell at a flow rate of 50 µL/min. In EC/MS experiments the reduced sample was directly introduced into the ESI-MS (LTQ – FT; Thermo Fisher Scientific, USA). The cell was operating in a pulse mode. The potential between the auxiliary and working electrode was set between 0 and minus 3000 mV to reduce the compounds of interest.

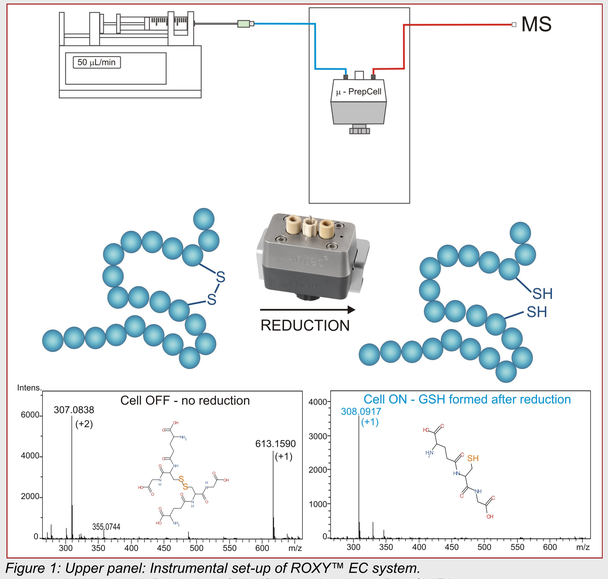

Figure 1: Upper panel: Instrumental set-up of ROXY™ EC system.
Middle panel: Reduction of disulfide bonds using µ-PrepCell™.
Bottom panel: Electrochemical reduction of GSSG to GSH.

Samples

Somatostatin (synthetic), Insulin (Bovine pancreas) and α–Lactalbumin (Bovine milk) were chosen as the model compounds for electrochemical reduction of disulfide bonds.

The peptides were selected to cover a broad range of molecular weights, 1637.88, 5731.61, 14176.81 Da, respectively.

Somatostatin is a 14 amino acids peptide, also called somatotropin release-inhibiting factor. Somatostatin has one intrachain disulfide bond that maintain the cyclic structure.

Insulin is a hormone produced by pancreas. Insulin is build by 51 amino acids forming two chains, A and B, and contains 3 disulfide bonds. Two bonds connect chain A and B and one intrachain disulfide bond is located on chain A.

α–Lactalbumin is 123 amino acids subunit of lactose synthase and may cause cow`s milk allergy in human. A globular structure of α–Lactalbumin is stabilized by four disulfide bonds (Cys25–Cys139, Cys47–Cys130, Cys80–Cys96, and Cys92–Cys110).

Figure 2: Sequence of Somatostatin, Insulin and α–Lactalbumin. The position of disulfide bonds are indicated.

Results

Insulin was used to show the efficiency of the electrochemical reduction of the disulfide bonds using the former Magic Diamond (MD) electrode and the newly developed S-S reducing electrode (Figure 3). For both electrodes, a square wave pulse was applied with optimized settings.

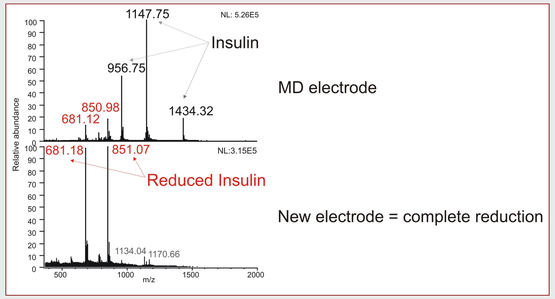

Figure 3: Reduction of Insulin on typically used MD electrode and a specially developed working electrode (Antec). The optimized pulse settings are applied.

Somatostatin

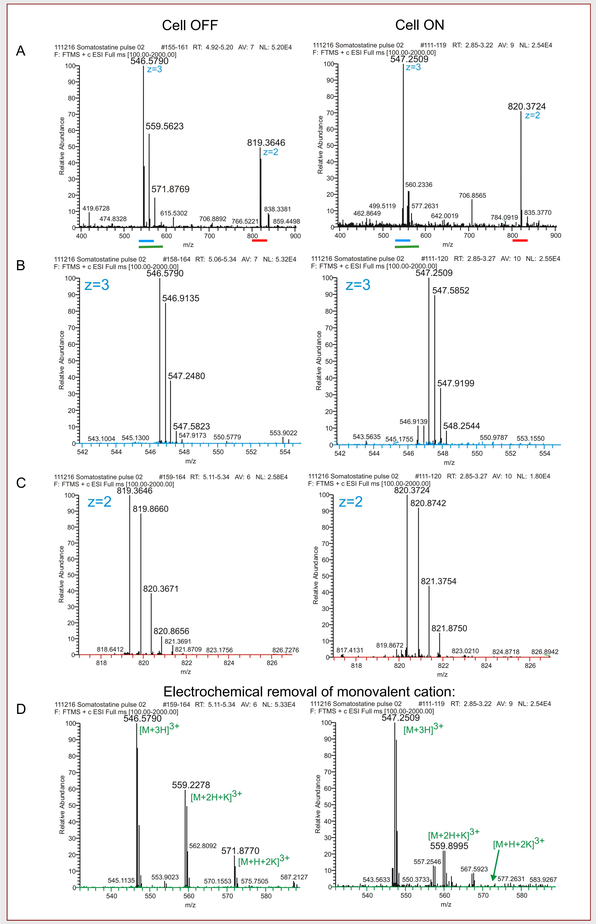

Figure 4: Electrochemical reduction of disulfide bonds in Somatostatin on a specially developed working electrode (Antec) by applying square wave pulses.
A) Full mass to charge range; B) Zoom of triply charged ions; C) Zoom of doubly charged ions; D) The removal of cation adducts as the result of applying square wave pulse.

Insulin

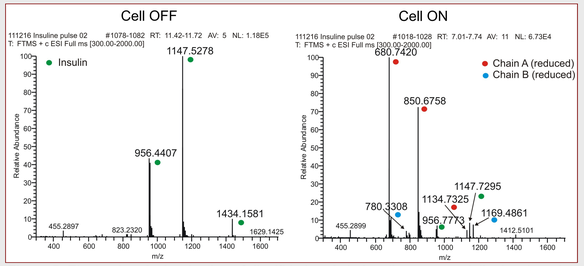

Figure 5: Electrochemical reduction of disulfide bonds in Insulin on a specially developed working electrode (Antec) by applying square wave pulses.

α–Lactalbumin

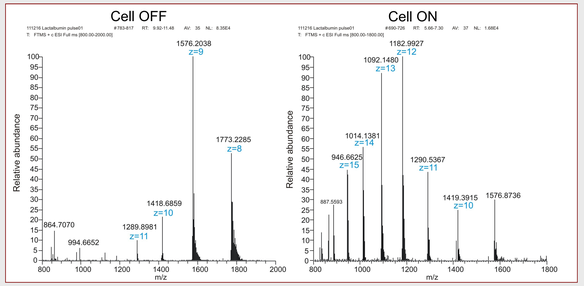

Figure 6: Reduction of α–Lactalbumin: shift of charge state distribution was observed suggesting unfolding due to S-S bond reduction.

Conclusions

In summary, we demonstrated new, electrochemically-based technique for efficient reduction of disulfide bonds in proteins and peptides. Compared to Magic Diamond (MD) working electrode with typical conversions rates of 20 to 30% the new proprietary S-S reducing electrode based on a semi-precious metal working electrode provides 100% reduction of the S-S bonds in peptides and proteins, opening new opportunities for faster and superior characterization of disulfide bonds in biopharmaceuticals.

Acknowledgements

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References

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