Introduction

Reduction followed by enzymatic digestion is a commonplace step in the characterization of protein therapeutics. Reduction is generally performed using chemicals such as TCEP at high concentrations, which interferes with the subsequent LC-MS analysis. Additionally, TCEP leads to very limited reduction efficiency when applied to proteins with a high disulfide bonds density. A novel method for the efficient and fast reduction of disulfide bonds in peptides and proteins combined with oxidative stress (OxS) and electrochemical (EC) reduction was presented. It allowed an alternative approach to traditional methods by using any reducing chemicals or enzymes and is purely instrumental resulting in a fully automated platform for fast characterization and assessment of peptide bonds in unprocessed biological samples.

Methods

In inclusion mode experiments (Fig 1B), typically 2 - 20 µM solutions of the target compound in 1% formic acid (FA) in water/methanol (50/10 or 50/50, v/v) were pumped through the EC cell; the voltage was either set at 50 or 100 mV. The outlet of the cell was directly connected to the ESI-MS.

Results - Insulin Reduction

Insulin was the first test compound to be reduced. Following optimization of the pulse settings to drive reduction through the EC cell into the MS, insulin was reduced in high yields into the two chains, A & B. The intra-chain disulfide bond was also reduced at high yield (Fig 3 A & B).

Cysteine-rich Hairpin & MAb Reduction

More complex proteins - Hepcidin, a cysteine-rich hairpin and Herceptin, a MAb - were also electrochemically reduced to demonstrate the capabilities of the EC cell. All disulfide bonds of Hepcidin were fully reduced, resulting in the almost complete sequencing of its peptide backbone while improving the back-exchange.

The MAb was efficiently and selectively reduced into LC & HC with only limited LC-HC, LC-2HC, LC-3HC combinations (Fig. 5), allowing the profiling of its major glycoforms. The reduction of the disulfide bridges is demonstrated by the shift of the charge state distribution based on the reduced number of disulfide bonds. The denaturation of these disulfide bonds could be reduced, one in the variable and one in the constant region.

Application to HDX Studies

The EC cell was positioned between the injector and the pepsin column outside the HDX manager (cell off position). The use of this set-up showed that the EC cell gave higher reduction yields than the traditional TCEP approach in only up to 1% FA, under standard HDX conditions. Decreasing both the TFA and temperature are not expected to jeopardize the reduction efficiency while improving the back-exchange.

Acknowledgements

This work would not have been possible without the collaboration and the work of the people Simon Nicoleck & Yo Van der Burgt at the Leiden University Medical Center (Netherlands) Simon Myking & Thomas Jørgensen at the University of Southern Denmark (Odense, Denmark).