On-line Disulfide Cleavage for Structural Characterization.

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ABSTRACT

A method is proposed to perform on-line LC reduction of disulfide containing analyte.

Integrated workflow to perform characterization of disulfide bonds with LC-MSMS system.

Eliminate need for alkylation

Eliminate risk of disulfide bond re-scrambling due to long term storage or incomplete alkylation

INTRODUCTION

Approaches to identify and characterize disulfide linkage in proteins have predominantly relied on protocols adapted from proteins sequencing methods. In these approaches, reduction is typically performed on the bench, requiring 15- to 30 minutes incubation. In the majority of cases, to avoid scrambling and recombination, alkylation is also performed requiring further incubation in some case under light controlled conditions. Though these protocols are well established and can be automated, they do add additional steps in the process that can lead to unnecessary analyte losses and require additional sample in the cases of disulfide bond characterization. In the current work, reduction can be done on column thus minimizing sample loss and reducing the number of steps in sample preparation.

MATERIALS AND METHODS

Sample Preparation:
Insulin analogues Humalogue® (Lilly) and Novolin® (NovoNordisk) were acquired from local pharmacy. Bovine insulin, bovine serum albumin (BSA), SiluMAB, dithiothreitol (DTT) and ammonium bicarbonate were acquired from Sigma (St Louis, MO). Protein digestion was performed using standard protocols where the DDT reduction and alkylation steps were removed.

HPLC Conditions:
LC was performed using a Shimadzu Nexera UFLC system operated at a flow rate of 400uL/min with a 1x75mm Poroshell column (Agilent) for the analysis of mAb and insulin analogues. For peptide analysis, the following columns were successfully used: Zorbax 2x100mm 3u (Waters) and Aries-C18-Peptide 2x150mm (Phenomenex). Gradients of water and acetronitrile, both with formic acid at 0.1%, were adjusted for each analyte.

MS/MS Conditions:
A SCIEX TripleTOF®5600 system with Turbo V™ source and Electrospray Ionization (ESI) probe was used. Generic source conditions and adjusted to the LC flow rate used.

On-Line Reduction

Reduction with DTT needs to occur at elevated pH (>7) and temperature (>60ºC) based on very early work performed by Cleland [1,2]. To meet the pH requirement, DTT (80mM) was prepared in a 500mM solution of ammonium bicarbonate, thus ensuring a change in pH on the LC column when a plug of 10-50uL is injected. This approach ensures that the pH conditions can change momentarily on the column for the reduction to occur, since the LC separation was performed at pH3. Also, to meet the second conditions of reduction, the LC column was constantly operated at elevated temperature (65-70ºC) . Figure 1 shows the workflow associated with on-line DDT reduction. In all cases, the analyte(s) to be reduced were pre-loaded on column.

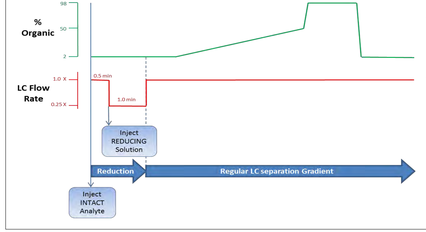


Figure 1.
Workflow for reduction of analyte on LC column. The analyte to be reduced is first injected on column and 'washed' isocratcaly for duration of 0.5min. Reducing solution, typically DTT (80mM) into bicarbonate solution, is injected at lower flow rate (0.25x of analytical flow rate) for 1min, after which the regular LC gradient condition and flow rate are applied. For non-reducing conditions, a blank DTT (0nM) solution is injected.

Insulin Reduction

All insulin analogues contain 3 disulfide links; 2 inter-chain and 1 intra-chain. As a model, bovine insulin was used to study the various parameter that could affect the efficiency of on-line reduction; temperature, flow rate, DTT injection volume. With this simple model protein, it is possible to monitor the various forms that can be produced by partial or complete reduction as depicted in Figure 2. Since all data was collected in MS mode at high resolution, all forms could be monitored. Figure 3 shows the effect of the DTT concentration on the efficiency of reduction of insulin. The overlay chromatogram for all species (intact, partially reduced and reduced) as well as heat-map plot show that increasing the amount of DTT on column leads to more complete reduction. Figure 4 shows the normalized response for each individual species monitored. Of note, the intact form is only observed when no-DTT is injected, whereas the completely reduced independent chains (a- and b-) optimize at elevated DTT amount (50uL of 80mM). At elevated DTT concentration, the insulin is essentially completely reduced.

Figure 2. Various form of insulin following partial or complete reduction. Sequence of bovine insulin is included.

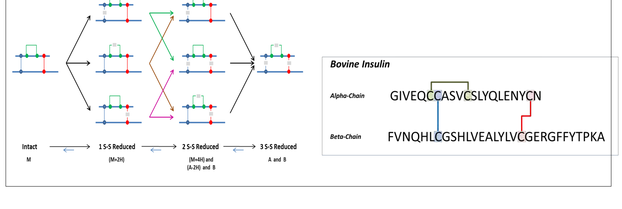


Figure 3. Effect of increasing DTT amount on column

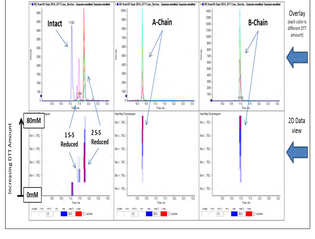


Figure 4. Normalized response versus DTT amount (A) and column temperature (B)

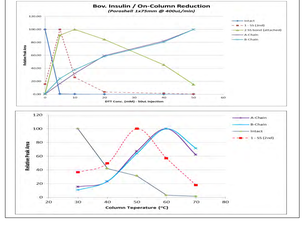
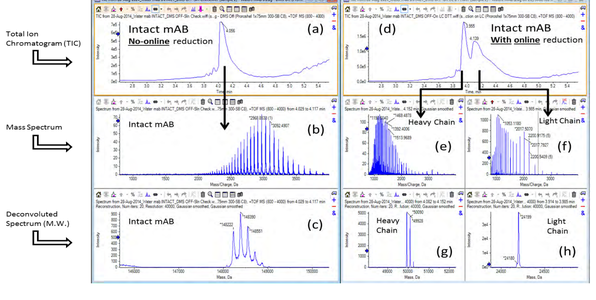


Figure 3 shows the effect of the DTT concentration on the efficiency of reduction of insulin. The overlay chromatogram for all species (intact, partially reduced and reduced) as well as heat-map plot show that increasing the amount of DTT on column leads to more complete reduction. Figure 4 shows the normalized response for each individual species monitored. As a function of DTT amount (A) and column temperature (B) Of note, the intact form is only observed when no-DTT is injected, whereas the completely reduced independent chains (a- and b-) optimize at elevated DTT amount (50uL of 80mM). At elevated DTT concentration, the insulin is essentially completely reduced. With respect to column temperature, values above 60oC will ensure more complete reduction of the insulin, but column capability should be considered with respect to this parameter. Many conventional C18 column, as well as Poroshell , have recommended limits ranging from 60 to 70ºC, a range that appears to be optimum for on-line reduction.

mAb Reduction

Figure 5(a) show the intact mAb analysis, as routinely done for mAb characterization. Figure 5(b) shows that the complete reduction of mAb can be obtained when using a temperature of 70ºC and a 30uL injection of 40mM DTT solution. The corresponding raw spectra and deconvoluted data can be found in 5(c) to (e) and 5(f) to (h), respectively. This approach offers on-line automation of reduction for full characterization of mAb. Similar results were observed for the mAb enzymatically cleaved at the Fc hinge using the IDES protease (data not shown).



Comprehensive disulfide bond reduction

To evaluate the use of this approach in the detailed characterization of disulfide bond in proteins. To evaluate this, BSA was used as model protein since it contains a total of 33 disulfide bridge. Digestion was performed without the reduction and alkylation steps. Figure 6, shows the sequence region from aa-141 to aa-211, the resulting expected peptides without and with reduction.. Figure 7 shows that all expected peptides, reduced and non reduced can be detected based on sequential injection performed without and with on-line reduction. This workflow offers the ability to perform a comprehensive characterization of the cross-linked region with disulfide bonds.

Figure 6. Peptide generated between aa-141/211 without and with on-line reduction

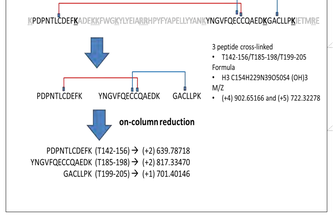
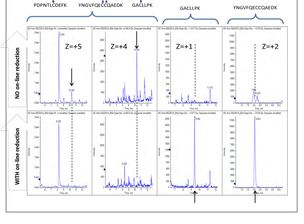


Figure 7. XIC for peptide observed without and with on-line reduction



CONCLUSIONS

The present work shows that on-column reduction of disulfide bonds can be performed in continuous flow mode. For insulin, complete reduction was achieved by injection of 40mM solution of DTT with column operated at 70ºC. This enables to analysis of non-reduced and reduced proteins or peptides with no additional sample preparation steps off-line for more complete characterization. In addition, the proposed approach alleviates the need to perform alkylation, a step that is associated with the use of light sensitive chemicals and necessary to avoid disulfide bond scrambling within the protein/peptide. This is particularly of concern if samples are left into the autosampler for long period of time prior to analysis. We demonstrated that the method could be applied to a broad range of compounds from tryptic peptides to intact mAbs. Though the present work was performed a limited set of LC columns (Poroshell, Zorbax and Aeries), it is anticipated that similar results could be obtained with other columns, as long as operation above 60ºC is possible for the stationary phase used. This work focused on the use of DTT for the reduction step, but it is expected that similar results with other reduction agent can be obtained. The proposed approach offer great flexibility for complete characterization of disulfide bond in complex samples or analyte.

REFERENCES

1 Cleland, W.W., *Biochemistry*, 1964; 3: 480–482
2 Whitesides GM, Lilburn JE, Szajewski RP. *J. Org. Chem.* 1977;42:332–338

TRADEMARKS/LICENSING

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