Systematic Comparison of Reduction and Alkylation Reagents reveals Side Chain Loss of Methionine due to Iodine-containing Alkylation Reagents

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OVERVIEW

Reduction and alkylation of S-S bonds is part of virtually any proteomics experiment. Despite its frequent use, only view studies (e.g. 1, 2) investigated the effects of reduction and alkylation procedures on the performance of peptide and protein identification. The published data indicate that iodoacetamide, which is the most frequently used chemical for the alkylation of S-S bonds, can result in massive side effects and may therefore influence the outcome of the experiment.

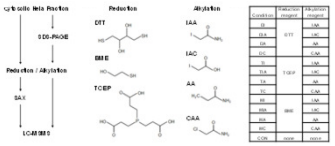
Using a cytosolic fraction of HeLa cells, we systematically evaluated different combinations of 3 common reduction reagents and 4 alkylation reagents using in-gel digests and SAX fractionated in-solution FASP digests (3). We found unspecific side reactions for all alkylation reagents which were especially pronounced for iodine-containing substances. Among these, we identified and evaluated the loss of the methionine side chain due to an efficient neutral loss in the gas phase as a major - and so far unrecognized - effect influencing peptide identification.

INTRODUCTION

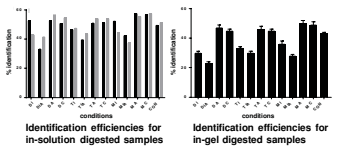
The reduction and alkylation of samples in proteomics experiments is a crucial step for the detection of cysteine containing peptides. Since the first studies performing in-gel and in-solution digestion of proteins introduced dithiothreitol (DTT) and iodoacetamide (IAA) for the reduction and alkylation of samples, little has changed with respect to the protocols most people use - the most frequent condition still is DTT/IAA. It has been shown that this combination can result in unspecific alkylation of residues other than cysteine (1, 2) and alternative substances have been introduced (4-6). These are, however, only used in ~20% of recently published studies. As unspecific side reactions have only been identified using standard proteins and synthetic peptides, it is questionable if the observed side effects are also detectable in real world samples. Therefore, this study is based on the cytosolic fraction of HeLa cells which should allow a realistic assessment of the degree of unspecific modifications present in proteomics experiments.

RESULTS 1 – COMPARISON OF REDUCTION AND ALKYLATION REAGENTS

For both in-solution and in-gel digested samples, we performed 12 different conditions for reduction and alkylation as well as one untreated control sample. We observed strong differences in numbers of identified peptide spectral matches (PSMs) when searching with standard search parameters (alkylation as fixed modification at Cys and oxidized Met) which was due to varying identification efficiencies of MSMS spectra. Alkylation efficiency was excellent for all conditions and numbers of Cys containing PSMs did not necessarily correlate with trends observed in the whole dataset.

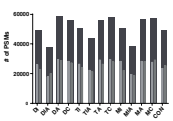


Workflow and combinations of reagents used for the study

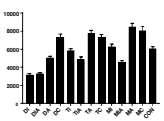


Identification efficiencies for in-solution digested samples

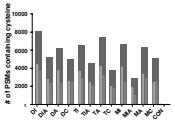
Identification efficiencies for in-gel digested samples



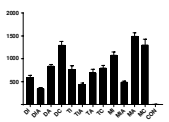
PSMs for in-solution digested samples



PSMs for in-gel digested samples



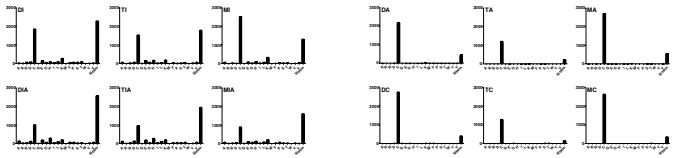
Identified Cys-containing PSMs for in-solution digested samples



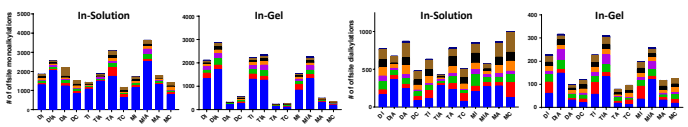
Identified Cys-containing PSMs for in-gel digested samples

RESULTS 2 – IDENTIFICATION OF SIDE REACTION PRODUCTS

Initial searches using a fraction of raw files and allowing for alkylation at all possible amino acids revealed alkylation of Tyr, Ser, Asp, Thr, His, Glu, Lys and the peptide N-terminus. These modifications where especially abundant for samples treated with iodine containing reagents. We also identified dimers of the alkylation reagents at these residues but no adducts of iodine. Even when adding numbers of all unmodified and modified PSMs for the different conditions, we were not able to achieve similar numbers for the different conditions.



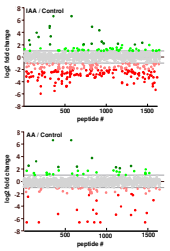
Amino acids annotated to be alkylated in combined results of several Mascot searches allowing for alkylation at all amino acid residues



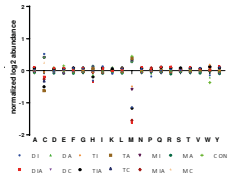
Amino acid dependent summed values of PSMs assigned to be alkylated with monomers or dimers of the respective alkylation reagent

RESULTS 3 – INVESTIGATION OF AMINO ACID COMPOSITION

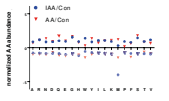
To further investigate differences in peptide identification, we performed 3plex dimethyl labeling of a sample alkylated with IAA vs. AA as well as a control sample. In peptides not containing cysteine we identified a >2 fold downregulation in 315 (IAA) and 70 (AA) peptides, respectively. When investigating the amino acid abundance in these peptides we noticed that 252 out of the 315 peptides contained Met which was not identified to be alkylated at all in the previous experiments. We then extended the analysis of amino acid composition to the other datasets which confirmed depletion of Met-containing PSMs for iodine-containing reagents.



Peptide ratios dimethyl labeling



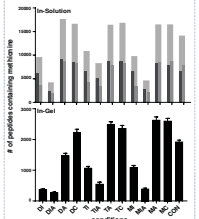
Amino acid abundance in all PSMs of in-gel digests



Amino acid abundance dimethyl labeling

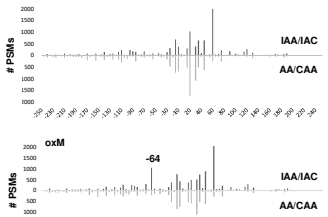
RESULTS 4 – DIFFERENCES IN IDENTIFIED METHIONINE PSMS

We compared the numbers of PSMs identified to contain Met for each of the datasets. It revealed that iodine-containing reagents resulted in a strong depletion of Met-containing peptides for both in-gel and in-solution digested samples.

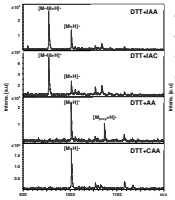


RESULTS 5 – METHIONINE CONTAINING PEPTIDES SHOW A LOSS OF 48DA

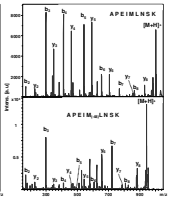
Since we were not able to identify any PSMs with alkylated Met residues, we searched for unexpected modifications. Error tolerant searches and unbiased mass tolerant searches did not result in any modifications identified in Met containing PSMs. After allowing for oxidation of Met (oxM) in mass tolerant searches, however, we were able to identify >1000 Met-containing PSMs unique for IAA and IAC at a mass value of -64 Da. This corresponds to the loss of the amino acid side chain from oxidized Met. We confirmed this in MALDI-MS experiments using a synthetic peptide.



Mass tolerant seraches for iodine-containing vs. Iodine free alkylation reagents. Shown are summed PSMs for bins of 5Da mass error



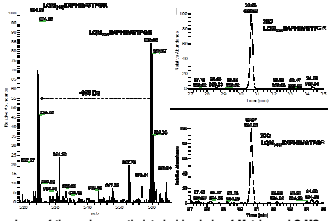
MALDI-MS analysis of the synthetic peptide APEIMLNSK reduced with DTT and alkylated with all different reagents



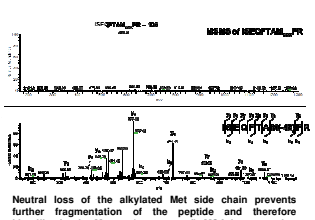
MALDI-MS/MS analysis of the synthetic peptide APEIMLNSK and the signal at [M+H-48]+

RESULTS 6 – LOSS OF 48 DA FROM METHIONINE IS DUE TO A NEUTRAL LOSS OF THE ALKYLATED SIDE CHAIN

We examined peptides which were annotated with a loss of 48 Da and searched manually for the intact alkylated Met residue. For both IAA and IAC, these signals showed perfect co-elution in the chromatogram proving the side chain loss to be due to a neutral loss in the gas phase. This loss was so efficient that it completely prevented the formation of other fragment ions and therefore peptide identification. This also explains why we were not able to identify any alkylated Met in the previous database searches. With a multi stage activation (MSA) method targeting this neutral loss, it was possible to generate spectra of excellent quality and to identify peptides with alkylated Met.



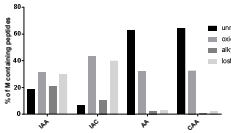
Loss of the carboxymethylated side chain of Met in an LC-MS run. The alkylated peptide and the one exhibiting the neutral loss show perfect co-elution



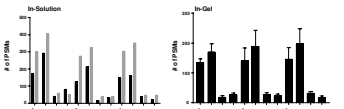
Neutral loss of the alkylated Met side chain prevents further fragmentation of the peptide and therefore identification by Mascot (upper panel). MSA fragmentation including MS3 of the neutral loss peak results in a rich fragmention ion specrum allowing for identification by Mascot (lowe panel)

RESULTS 7 – ANALYSIS OF METHIONINE ALKYLATION

By analysis of in gel-digested samples with a MSA method and reprocessing of the initial files we identified abundant modification of methionine residues by alkylation for the iodine containing reagents.



Data obtained from an analysis with a Met side chain loss triggered MSA method (all samples were reduced with DTT)



Identification of in-source fragmented peptides with alkylated Met residues using the loss of Met side chain as variable modification

METHODS

HeLa cells were grown in 15 cm dishes using DMEM supplemented with FCS, Penicillin, and Streptomycin until confluency, harvested by scraping and lyzed in sucrose buffer with a dounce homogenizer. The resulting lysate was cleared by subsequent centrifugation steps at 1,000; 10,000 and 100,000 x g and the supernatant collected as the cytosolic fraction. Proteins were acetone-precipitated, re-solubilized in SDS buffer and the protein content determined. For in solution digests, samples were reduced and alkylated using the different combinations followed by tryptic digestion on 30 kDa molecular weight cutoff spin filters following the FASP approach (3). Resulting peptides were fractionated using strong anion exchange (SAX) centrifuge tips (3). For in gel digestion, unreduced samples were separated using SDS-PAGE, stained using Coomassie and a representative section of the gel between 30 and 50 kDa was excised and cut to pieces. Reduction and alkylation was performed using the different reagent combinations in the gel followed by a washing and dehydration step and tryptic digestion overnight. Resulting samples were analyzed by LC-MSMS using an Orbitrap Velos mass spectrometer. Database searching was performed with Proteome Discoverer in combination with Mascot for known modifications. For unknown modifications, error tolerant (7) and mass tolerant (8) searches were carried out using the Mascot search interface.

REFERENCES

(1) Boja E. S. et al. 2001, Anal. Chem. 73, 3576-3582; (2) Yang Z. H. et al. 2007, J. Mass Spectrom. 42, 233-243; (3) Wisniewski, J. R. et al. 2009, J. Proteome Res. 8, 5674-5678; (4) Sechi S. et al. 1998, Anal. Chem. 70, 5150-5158 (5) Liu F. et al. 2016, J. Proteome Res. 15, 4666-4674; (6) Paulech J. et al. 2013, BBA-Proteins Proteomics 1834, 372-379; (7) Creasy D. et al. 2002, Proteomics 2, 1426-1434; (8) Chick J. et al. 2015, Nature biotechnology 33, 743-749.