Optimization of a 2DE-Based Biotin Switch Method for Proteomics Analysis of Nitrosylated Proteins

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Abstract

The biotin switch assay (BSA) has been widely used to detect cysteine S-nitrosylation within proteins[1]. In this study, we confirmed the specificity of ascorbate towards the reduction of S-nitrosylated peptides. In addition, we have used biotin-maleimide instead of biotin-HPDP for the quantification of cellular nitroproteome changes by 2DE.

Introduction

BSA has been widely used for the identification of *S*-nitrosylated proteins. In this method, free thiols are first alkylated with methylmethanethiosulfonate (MMTS) followed by nitrocysteine (SNO) reduction by ascorbate, to generate fresh thiols which are then alkylated with biotin-HPDP (Fig. 1) for surrogate SNO detections (Fig. 2). Despite its wide adaptation, the specificity and efficiency of this method has recently been challenged by the possibility of producing false-positive results due to reduction specificity[2]. Furthermore, it is also challenging to study SNO proteins via 2DE-based approach because biotin-HPDP could be removed by the reducing agents employed in typical 2DE buffers (Fig. 1). In this study we aim to address two questions: firstly whether ascorbate specifically reduces SNO but not disulfides; and secondly, whether biotin maleimide (biotin M, Fig. 1) can replace biotin-HPDP for 2DE-based SNO proteome analysis

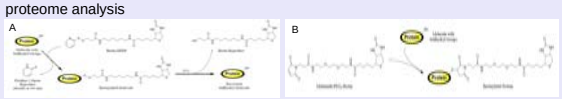
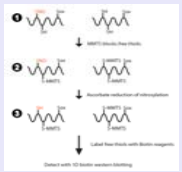


Figure 1. Reaction scheme for biotinylation of sulfhydryl molecules with biotin-HPDP (A) and biotin-PEG$_2$-maleimide (B). (From Thermo scientific).



Specific aims:
1. Does ascorbate efficiently reduce nitrosothiols?
2. Does ascorbate reduce disulfide bonds?
3. Can biotin-M replace biotin–HPDP for SNO proteome analysis via 2DE?
4. Can SNO peptide or biotin labeled peptides be effectively analyzed by MALDI or ESI MS?

Figure 2. The biotin switch assay. Modified from Jaffrey *et. al.*[1].

Methods

1. Cellular SNO content assay

HeLa cell proteins were treated with 100 µM nitroso-glutathione (GSNO) at 37ºC for 30 min in the dark to produce SNO proteins. The proteins were precipitated and washed with cold acetone to remove excess GSNO. The protein pellet was dissolved in a biotinylation buffer[1] and treated with 1 to 50 mM of ascorbate at rt for 1 h. S-nitrosothiol contents were determined using Saville assay[3].

2. MALDI and ESI MS detection of SNO and biotinylated peptides

(a) A synthetic human caspase 3 peptide (casp3$^{163\text{-}175}$,163-CRGTELDCGIETD-175) containing two cysteines was reduced with either ascorbate or DTT, and labeled with either biotin-HPDP or biotin M to detect cysteine thiols. The biotinylated peptide were analyzed by ABI 4800-MALDI-TOF-TOF MS.

(b) Reduced casp3$^{163\text{-}175}$ was nitrosylated with 10 x GSNO. The remaining free thiols were alkylated by MMTS. The modified peptide was analyzed by QTOF-MS[4].

3. 1D and 2D analyses of biotin-HPDP and biotin M labeled SNO proteins

HeLa or SH-SY5Y cell proteins were nitrosylated with 100 µM of GSNO and then processed for BSA with either biotin-HPDP or biotin M. Biotinylated proteins were detected by either 1D or 2D Western blotting. Alternatively, biotinylated proteins were also affinity enriched using streptavidin beads and analyzed by 2DE.

Results

1. Nitrosylated proteins can be readily reduced by ascorbate

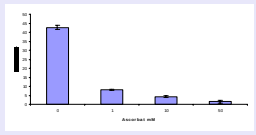


Figure 3 SNO content assay of HeLa cell proteins. The proteins were first pretreated with 100 µM of GSNO, and then treated with increasing concentrations of ascorbate. Ninety percent of SNO from HeLa cell proteins could be reduced by just 1 mM ascorbate at rt for 1 h.

2. Ascorbate can not reduce disulfide bonds under certain conditions

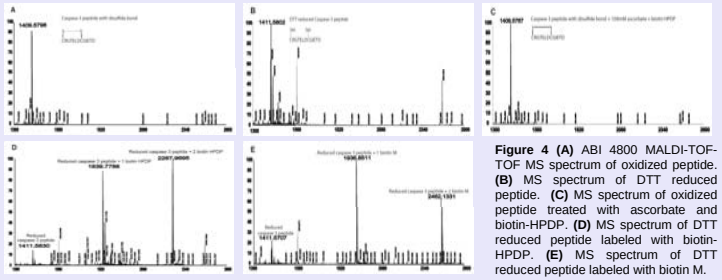


Figure 4 (A) ABI 4800 MALDI-TOF-TOF MS spectrum of oxidized peptide. **(B)** MS spectrum of DTT reduced peptide. **(C)** MS spectrum of oxidized peptide treated with ascorbate and biotin-HPDP. **(D)** MS spectrum of DTT reduced peptide labeled with biotin-HPDP. **(E)** MS spectrum of DTT reduced peptide labeled with biotin M.

3. S-nitrosylated caspase 3 peptide can be directly analyzed by ESI MS

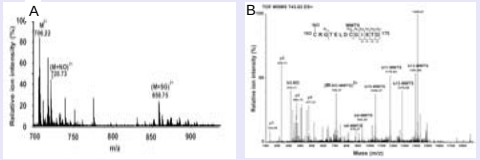


Figure 5 (A): Waters QTOF ESI MS spectrum of Caspase 3 peptide after GSNO treatment. A doubly-charged ion (*m/z* 720.73) corresponding to the S-nitrosylated peptide was observed (B): MS/MS of MMTS alkylated and nitrosylated caspase 3 peptide for the localization of SNO site.

4. Biotin M can replace biotin-HPDP for 1D Western blot nitroproteome analyses

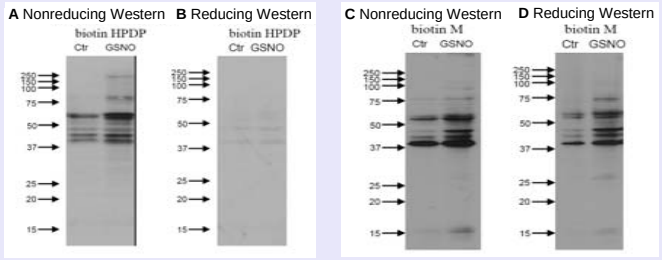


Figure 6 BSA of HeLa cell proteins labeled with biotin-HPDP and biotin maleimide. Total proteins from HeLa were extracted and in vitro nitrosylated. Western blotting were performed with 12.5% non-reducing and reducing SDS-PAGE. Biotin-HPDP labeled proteins in nonreducing (**A**) and reducing (**B**) Western blotting. Biotin M labeled proteins in non-reducing (**C**) and reducing (**D**) Western blotting. DTT reduction removed all the biotin-HPDP labels prior to electrophoresis.

5. Biotin M is more effective than biotin–HPDP for 2DE nitroproteome analyses

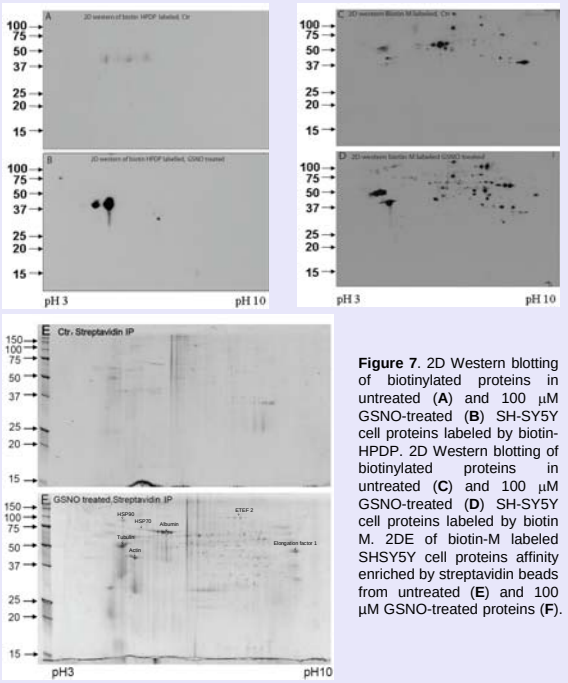


Figure 7. 2D Western blotting of biotinylated proteins in untreated (**A**) and 100 µM GSNO-treated (**B**) SH-SY5Y cell proteins labeled by biotin-HPDP. 2D Western blotting of biotinylated proteins in untreated (**C**) and 100 µM GSNO-treated (**D**) SH-SY5Y cell proteins labeled by biotin M. 2DE of biotin-M labeled SHSY5Y cell proteins affinity enriched by streptavidin beads from untreated (**E**) and 100 µM GSNO-treated proteins (**F**).

Conclusions

1. Ascorbate readily reduced SNO's but not disulfide bonds in BSA method.
2. S-nitrosylated peptide can be directly analyzed by ESI-QTOF MS.
3. Biotin maleimide adds 525 amu to a peptide mass. Biotin-HPDP adds 428 amu to a peptide mass.
4. Biotin-HPDP labeled proteins can not be detected in reduced SDS PAGE and 2DE
5. Biotin maleimide can replace biotin-HPDP for 2DE based nitroproteome analyses.

Acknowledgement

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References

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