

Characterization of protein biotinylation sites by peptide-based immunoaffinity enrichment

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Introduction

Biotin labeling in combination with LC-MS/MS has been widely applied in large-scale analysis of protein post-translational modifications, cell surface proteins, protein-protein interactions and protein subcellular localization. Direct characterization of protein biotinylation sites is still challenging due to the low recovery of biotinylated peptides using conventional streptavidin/avidin based purification method. It has been found that anti-biotin antibody is a better capture reagent for biotinylated peptides compared to streptavidin/avidin. In this study, we established an immunoaffinity enrichment method using a monoclonal anti-biotin antibody and also compared it to approaches from two published papers using polyclonal antibodies from various vendors. We then demonstrated our enrichment method by applying it to characterization of protein biotinylation sites from APEX proximity labeling in living cells.

Methods

Trypsin digested mouse liver peptides were labeled with EZ-link NHS-biotin and used as the test sample for method optimization and comparison. HEK 293T cells stably expressing fused β 2AR and APEX were cultured, incubated with biotin phenol, and treated with agonist BI167107 for 10 min. Protein biotinylation was activated by adding H₂O₂. Cells were harvested and digested with trypsin. Tryptic peptides were immuno-purified by beads coupled with anti-biotin antibodies. Biotinylated peptides were eluted from beads, desalted, and finally analyzed on Thermo Scientific Q Exactive or Fusion Lumos. Identification of biotinylated peptides was conducted by Sequest searching. MS1 Label-free quantitation of biotinylated peptides was performed by Skyline.

Results

The method comparison experiment is composed of two technical analyses (including immuno-precipitation and LC-MS/MS) of biotin labeled mouse liver peptides using different antibodies and protocols. An average of 3425(\pm 227) unique biotinylated peptides were identified using our established method, compared to 3245 \pm 88, 1425 \pm 129, and 193 \pm 20 biotinylated peptides using reported approaches in the literature. The demonstrative experiment for the utility of our method uses proximity dependent labeling strategy by APEX peroxidase for characterization of interacting protein sites of β 2AR in HEK293T cells. In total 1354 unique biotinylated peptides from 125 proteins were identified and quantified. Among them, the levels of 148 biotinylated peptides from 125 proteins were significantly changed (fold change \geq 2.5) responding to the treatment of the agonist.

Novel aspect

We describe a robust and well-performing immuno-enrichment method for biotinylated peptide

Background

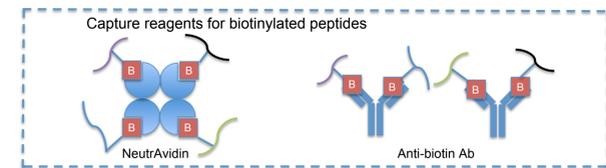
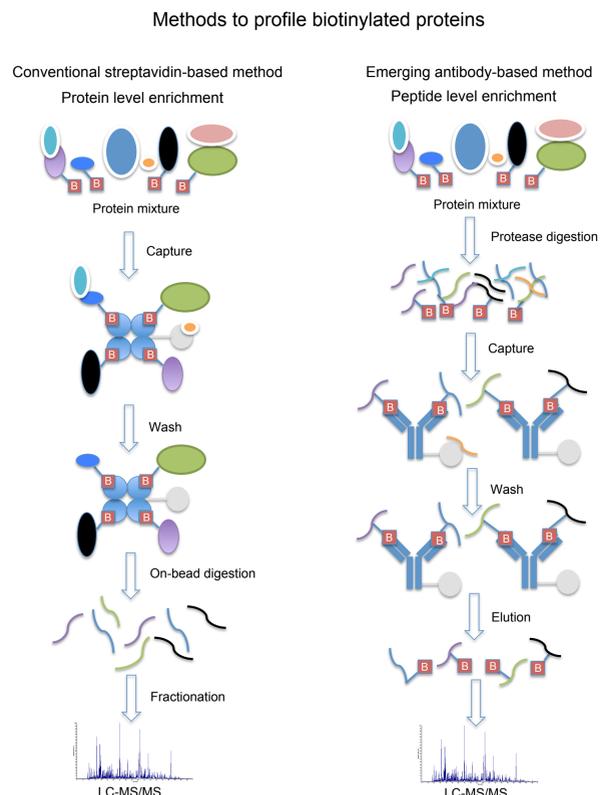


Figure 1. Overview of conventional and emerging methods for protein biotinylation identification. Conventional method applies streptavidin beads to purify biotinylated proteins. After background proteins were washed off under denaturing condition, biotinylated proteins retained on bead are digested by protease. Then nonbiotinylated peptides are eluted and subsequently analyzed by LC-MS/MS. Emerging method focuses on identifying biotinylated peptides. First proteins are digested into peptides and then biotinylated peptides are captured by bead conjugated antibody. Advantages of purifying biotinylated peptides include determination of biotinylation sites and limitation of false positives which are usually non-specific bound proteins and naturally biotinylated proteins. Choosing the right capture reagent is the key of peptide level purification. There are mainly two types of reagents available (dashed rectangle). In 2014, Schiapparelli et al described a method using neutravidin, a deglycosylated avidin, to enrich biotinylated peptides¹. In 2017, Kim et al² and Udeshi et al³ reported methods based on anti-biotin antibody enrichment of biotinylated peptides. Anti-biotin Antibody is a better capture reagent than neutravidin for biotinylated peptides because of its lower binding affinity to biotin which boosts efficient elution of biotinylated peptides.

Results

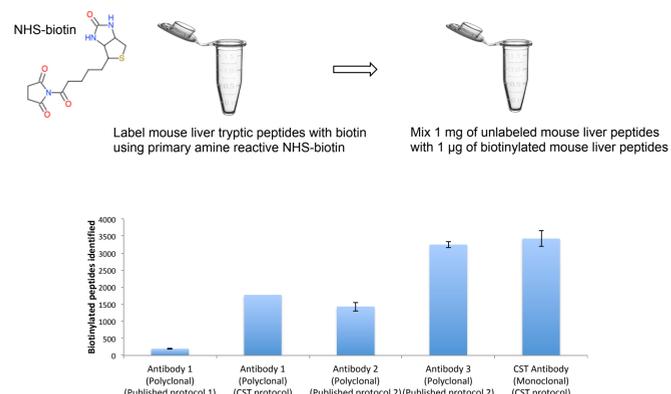


Figure 2. Comparison of different anti-biotin antibody-based methods for biotinylated peptide identification. Mouse liver peptides were chemically biotinylated and 1 µg was mixed with 1 mg of untreated mouse liver peptides for each sample. Enriched peptides from each immuno-affinity enrichment were analyzed by Orbitrap Q Exactive and identified by SEQUEST protein database search. Published methods using different commercially available anti-biotin antibodies and immuno-enrichment protocols were compared with Cell Signaling Technology (CST) method which applies CST rabbit monoclonal anti-biotin antibody (A7C2A) and an optimized immuno-precipitation protocol. (CST IP steps: 1. Wash bead coupled antibody with 1X PBS four times 2. Incubate antibody-bead with peptides for 2 h at 4 °C 3. Wash by 1X immuno-affinity purification (IAP) buffer two times, followed by DI water three times 4. Elute biotinylated peptides by 80% ACN/0.2% TFA)

Table 1. Comparison of polyclonal antibodies and monoclonal antibodies

| Polyclonal antibody | Monoclonal antibody |
|---|---------------------------------|
| Mixed population of antibodies | Single population of antibodies |
| May bind to off-target epitopes | Only bind single specific site |
| Limited resource | Renewable resource |
| Different lots will differ in performance | Uniform in performance |

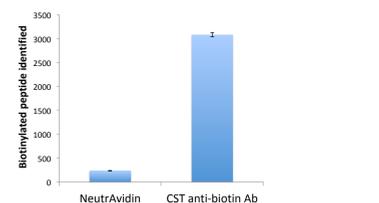


Figure 3. Comparison of different capture reagents for biotinylated peptides. Mouse liver peptides were chemically biotinylated and 1 µg was mixed with 1 mg of untreated mouse liver peptides for each sample. Biotinylated peptides were enriched by either NeutrAvidin or anti-biotin antibody (A7C2A). Enriched biotinylated peptides were analyzed on an Orbitrap Q Exactive instrument and MS/MS spectra were searched by SEQUEST.

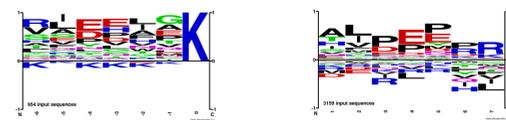


Figure 4. Motif analysis done using all biotin peptides enriched and identified from chemically biotinylated mouse liver peptides. The motif logos for lysine residues (left) and peptide N-termini (right) show that the anti-biotin (A7C2A) antibody is a general biotin antibody that recognizes the biotin modification and does not demonstrate discernable sequence preference.

Application

Proximity labeling

Proximity labeling in living cells, such as APEX or BioID, is an emerging technology which determines protein-protein interaction and protein subcellular localization. Compared to conventional methods such as co-immunoprecipitation of protein complexes, it has the advantages of eliminating false positives and preserving weak or transient interactions. Our demonstrative experiment for the utility of BiotinScan® is characterization of interacting proteins of β 2AR in HEK293T cells using engineered ascorbate peroxidase (APEX).

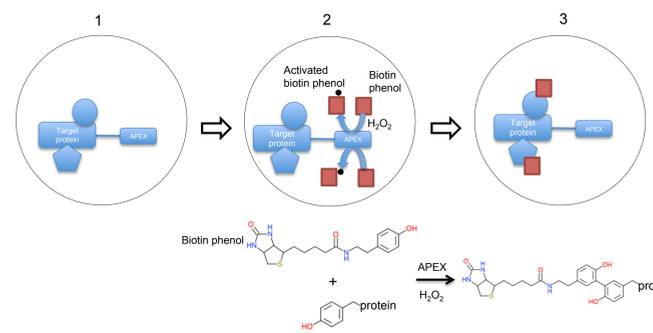


Figure 5. Overview of APEX proximity dependent labeling technology. Step 1. Co-expression the protein of interest with APEX peroxidase. Step 2. Activation of biotinylation reaction by adding biotin phenol and H₂O₂. Step 3. Labeling of nearby interacting proteins by reactive biotin phenol. The chemical mechanism which depicts the labeling of biotin phenol with protein tyrosine side chain is also shown.

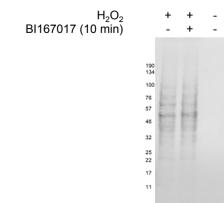


Figure 6. Biotinylated proteins from the β 2AR-APEX proximity labeling experiment blotted by an anti-biotin antibody (CST #5597). β 2AR-APEX cells were cultured and treated with 100 nM of β 2AR agonist BI167107 for 10 min. Untreated cells and treated cells were labeled with biotin by adding biotin phenol and H₂O₂. One plate of untreated cells were left without H₂O₂ treatment. The reactions were quenched and proteins were extracted by urea buffer. The western blot shows that observed protein biotinylation was directly from proximity labeling.

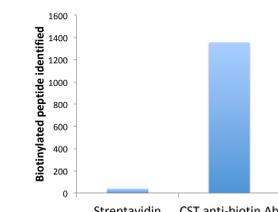


Figure 7. Comparison of biotinylated peptides identified from anti-biotin antibody (A7C2A) enrichment of biotinylated peptides and that from Paek et al.⁴ who used streptavidin-based enrichment method for biotinylated protein identification. In both studies, β 2AR-APEX co-expressed HEK293T cells were cultured. Proximity labeling was activated by adding biotin phenol and H₂O₂ which biotinylated protein tyrosine residues near β 2AR-APEX. After enrichment, peptides were analyzed by Thermo Fusion Lumos and MS/MS spectra were searched by SEQUEST.

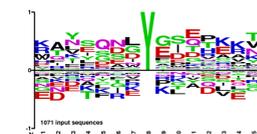
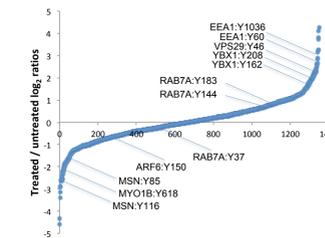


Figure 8. Motif analysis done using all biotinylated peptides identified from β 2AR-APEX cells. The motif logo for lysine residues show that the anti-biotin (A7C2A) antibody is a general biotin antibody that recognizes the biotin modification and does not demonstrate discernable sequence preference.



| Proteins | Biotinylated Peptides | Site | Treated/Untreated Anti-biotin | Treated/Untreated Streptavidin ⁴ |
|----------|-------------------------------|------|-------------------------------|---|
| VPS29 | ESYDYDK | 46 | 5.6 | 6.3 |
| ARF6 | NWYVQPSCATSGDGLYEGLTSLTSNYK | 150 | -2.3 | -3.1 |
| RAB7A | NALKQETVELYINFFPEPIK | 183 | 2.1 | 4.0 |
| RAB7A | FSNQYK | 37 | -1.1 | 4.0 |
| RAB7A | ADAWCYSK | 144 | 1.9 | 4.0 |
| EEA1 | HYEAVHDAGNDSHGGSNLALK | 60 | 7.4 | 23.4 |
| EEA1 | QLQSDFYGR | 1036 | 8.9 | 23.4 |
| YBX1 | NYQQNYQNSSEGEKNEGSAPFGAQQR | 162 | 3.8 | 3.7 |
| YBX1 | RPQYSNPQVQGEVMEGADNQGAEGQRPVR | 208 | 4.3 | 3.7 |
| MYO1B | YVLGLENVR | 618 | -4.6 | -3.6 |
| MSN | EGLNDDIYCPPEAVLLASYAVQSK | 116 | -6.1 | -3.4 |
| MSN | FYPEDVSEELIQDITQR | 85 | -3.1 | -3.4 |

Figure 9. Relative abundances of identified biotinylated peptides from BI167017 treated and untreated β 2AR-APEX cells. The dot graph shows that 1354 unique biotinylated peptides corresponding to 858 proteins were identified and quantified from anti-biotin antibody (A7C2A) purification. Among them, levels of 148 biotinylated peptides from 125 proteins were significantly changed (\geq 2.5) responding to the agonist. Examples of identified biotinylation sites and proteins were marked. The table lists the corresponding biotinylated peptides and the ratios between treated and untreated for these examples. Fold change results from Paek et al.⁴ who used streptavidin purification for these proteins were also shown for comparison. The ratios from published data were based on proteins. We assume peptides from one protein have same fold changes as the protein.

Conclusions

- The emerging method using antibody to purify biotinylated peptides is better than conventional method utilizing streptavidin to enrich biotinylated proteins at identification of biotinylation sites. Rabbit monoclonal anti-biotin antibody (A7C2A) from Cell Signaling Technology shows advantages over other commercially available polyclonal antibodies and NeutrAvidin reagent.
- Anti-biotin antibody (A7C2A) from Cell Signaling Technology can be applied in identifying biotinylated peptides and determining protein binding partners in APEX proximity labeled cells. Besides, it can be used in any other large-scale study of protein biotinylation sites, such as characterization of biotin-labeled protein post-translational modifications and profiling of biotin-labeled cell surface proteins.

The PTMScan® Anti-Biotin Kit (CST #41343) includes PTMScan® anti-biotin (A7C2A) immuno-affinity beads, PTMScan® IAP buffer, and protocol. It enables customers to perform purification of biotinylated peptides in house.

References

- Schiapparelli et al. J Proteome Res. 2014 13(9): 3966-78
- Udeshi et al. Nat Method 2017 14(12):1167-1170
- Kim et al. J Proteome Res. 2018 17(2): 759-769
- Paek et al. Cell 2017 169(2): 338-349

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