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Efficient approach for profiling photoaffinity labeled peptides with a cleavable biotinyl photoprobe

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ABSTRACT

Based on the application of our recent biotinyl photoprobe with a cleavable *N*-acylsulfonamide, an efficient process has been developed for profiling photoaffinity labeled peptides among a large excess of unlabeled concomitants. *N*-acylsulfonamide group was found to be stable under the usual S-pyridylethylation condition of cysteine residues whereas the group was easily cleaved by N-alkylation with iodoacetic acid in acidic condition. The selective nature between two common protein alkylation reactions was evaluated with L-glutamate dehydrogenase (GDH) using an acidic amino acid photoprobe with biotinylated acylsulfonamide function. The labeled GDH was successfully subjected to S-pyridylethylation keeping the biotin tag intact, and then was easily released from streptavidin matrix with high purity via iodoacetic acid-mediated alkylation under mild condition at pH 5.0.

One of the major difficulties for the identification of photolabeled sites within a target protein is the inefficiency of cross-linking, although photoaffinity labeling (PAL) has been recognized as a useful chemical tagging technique that can be accessible to low-affinity or membrane-bound proteins.[1] The low labeling yield enforces the laborious isolation steps for retrieving labeled products from a large excess of complex concomitants. Although the use of benzophenone among useful photophores usually increases the yield, the cross-linking was reported to require prolonged irradiation and to be limited to a few amino acid residues such as methionine, histidine.[2] The photochemical properties of aryldiazirine is superior to arylazide in terms of stability of the cross-link and wavelength for excitation,[3] the labeling yield is usually low due to the rapid quenching of reactive carbene with the surrounding materials. Owing to the advance in specificity and stability of carbene-mediated label, photoprobes with biotinyldiazirines were developed for fishing out the labeled product by trapping on avidin matrices.[4] A great deal of effort have been devoted to introduce scissile functions in biotinyl probes which are labile in photochemical[5] or enzymatic[6] reactions for the recovery of captured products from tightly associated avidin–biotin complex whereas no simple and efficient method has been established for the extensive use of PAL technology. Recently, we found that the *N*-acylsulfonamide group was cleaved under the mild condition of well-established protein S-carboxymethylation.[7] Here we report an efficient purification process which consists of selective protein S-alkylation, the

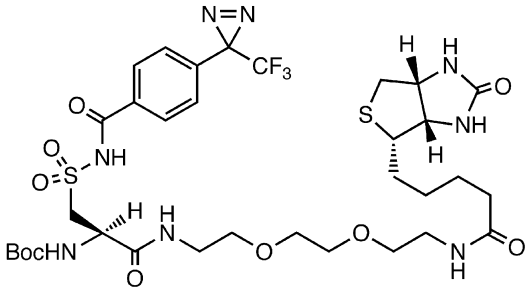
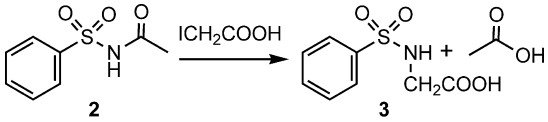


Figure 1. Structure of the photoprobe.



pH	8.5	5.0
Yield of **3** (%)	43 ± 2	35 ± 2

Figure 2. pH-dependence of N-alkylation/cleavage reaction with iodoacetic acid. The reaction was carried out with 10 mM of iodoacetic acid and incubated in an appropriate buffer solution at 25 °C for 8 h (0.1 M Tris–HCl for pH 8.5, 0.1 M sodium acetate for pH 5.0). The values are total yields of two reaction steps and represent the average of three assays.

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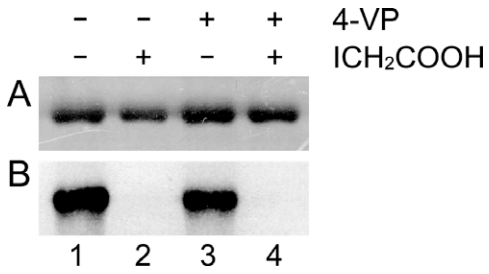

Figure 3. Discrimination between N- and S-alkylation in an acidic condition. After gel filtration of photoproducts of GDH and probe **1**, the sample was subjected to treatment in acetate buffer (pH 5.0). Lane 1: the sample without any treatments; lane 2: the mixture treated with iodoacetic acid (200 mM); lane 3: the mixture treated with 4-VP (23 mM); lane 4: the sample in lane 3 treated with iodoacetic acid (200 mM). Panel A: gel visualized with Coomassie Brilliant Blue (CBB), panel B: chemiluminescence detection utilizing avidin-HRP conjugate after blotting onto PVDF membrane.

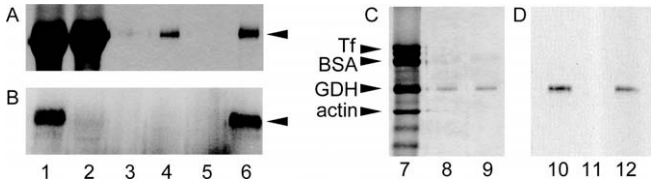

Figure 4. Purification of labeled GDH by cleavage from avidin matrix. The photoproducts were filtrated on G-50, pyridylethylated, trapped into streptavidin-agarose beads, and then cleaved with iodoacetic acid (200 mM in 0.5 M acetate buffer pH 5.0) in the presence of 0.2% SDS. Panels A and B: photolabeling of GDH with the probe **1**, and panels C, D: a protein mixture consisting of transferrin, BSA, GDH and actin was photolabeled. The samples were visualized by silver stain in panels A and C, or by chemiluminescence utilizing a biotin tag in panels B and D, respectively. Lanes 1, 7, 10: the sample after photolysis, lane 2: supernatant after the treatment of avidin-agarose, lane 3: supernatant after repeated washing process for five times, lanes 4, 8, 11: cleaved product, lane 5: heat eluted sample from matrix of lane 4, lanes 6, 9, 12: heat eluted sample from matrix without cleavage.

specific biotin extraction of labels on avidin matrix, and the selective acylsulfonamide cleavage for releasing labeled proteins. The method has been evaluated with bovine L-glutamate dehydrogenase (GDH, EC 1.4.1.2-3) and the probe described in Figure 1.[7]

We first examined the cleavage of simple *N*-acetylbenzenesulfonamide **2** at different pH conditions using iodoacetic acid, a common S-alkylation reagent, because the pK_a value of general acylsulfonamide is closed to that of carboxylic acid. The values in Figure 2 represented the percentage of cleavage products, determined by HPLC analysis. While the alkylation-cleavage steps with iodoacetic acid proceeded more efficiently in alkaline solution, the N-alkylation at pH 5.0 still provided 35% yield of cleavage product that is comparable value to 43% yield at pH 8.5. This property will give a great advantage to avoid the undesired side reactions during the iodoacetic acid-mediated cleavage of labeled proteins since it is well known that the basic conditions increase the possibility for modifications of amino acid residues like tyrosine, histidine, lysine, and even methionine during S-carboxymethylation of protein.[8]

The acidic condition of cleavage established in the model system was evaluated at the protein level with the photoaffinity labeled GDH protein. Since the reductive S-alkylation of proteins prior to the digestion is the due course for the identification of labeled sites, we investigated S-alkylation with 4-vinylpyridine (4-VP) as another type of common reagent to accomplish the selective blocking of cysteine SH group without the cleavage at the acylsulfonamide linkage. The chemiluminescence detection of SDS–PAGE separated and electro-blotted GDH samples onto poly(vinylidene difluoride) (PVDF) showed specific incorporation of biotin tag on the protein band near 50 kD (Fig. 3, lane 1). The chemiluminescence due to the photo-incorporated biotin was disappeared after the alkylation with iodoacetic acid (lane 2) whereas the signal was retained upon the treatment with 4-VP (lane 3). The results clearly indicate the development of simple method for efficient isolation of photoaffinity biotinylated proteins with the cleavable approach, which is the sequence of procedures with the selective protein S-alkylation, the specific biotin purification of labels on avidin matrix, and the selective acylsulfonamide cleavage.

For profiling photolabeled peptides, the pyridylethylated mixture of GDH was subjected to the purification with streptavidin–agarose (Fig. 4). After washing to remove the unbound protein (lane 3), the photolabeled GDH protein was recovered using iodoacetic acid in acetate buffer at pH 5.0 (lane 4). The sample cleaved from the matrix did not show the emission due to the loss of biotin moiety. The recovery yield was 84% of labeled proteins (lane 4, from the concentration of silver stain in lane 6) and that is compa-

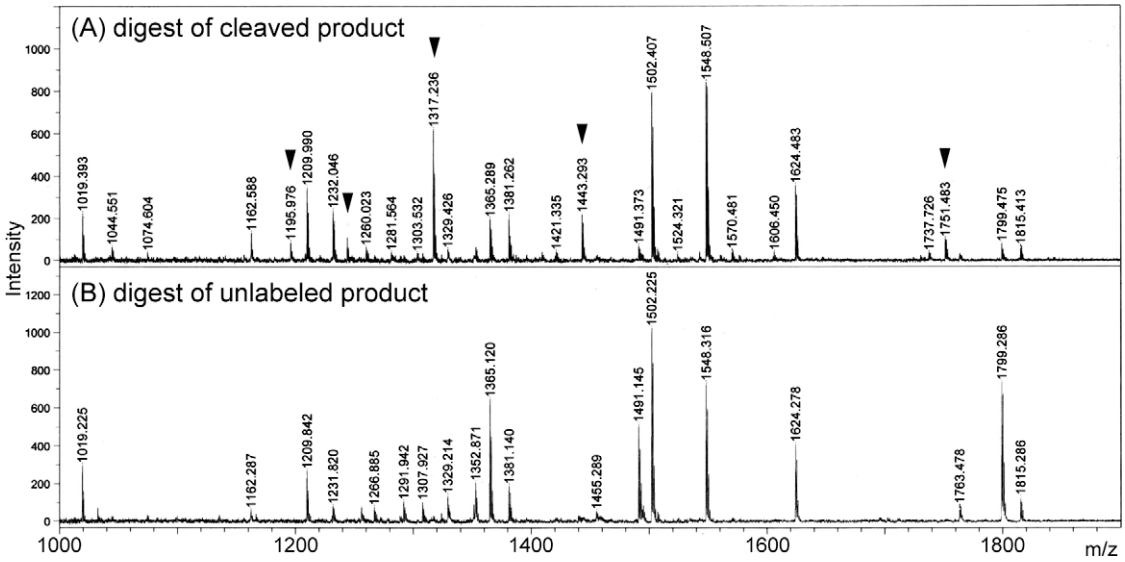

Figure 5. MALDI-TOF mass spectra of peptides from Lys-C digest of labeled GDH protein purified by direct cleavage (A) and the GDH protein after the same treatment without the probe (B), respectively. Arrows indicate the specific peaks in the digest from cleaved sample.

rable to the yield obtained by a harsh heat elution (93%, lane 6, calculated from the emission intensity in lanes 1 and 6).[9] The panels C and D shows that purification of the labeled GDH with high quality has been also achieved by performing PAL of a protein mixture consisting of transferrin, BSA, GDH, and actin.[10] The recovered GDH was further purified on SDS–PAGE and the GDH band was subjected to the in-gel digestion with lysyl endopeptidase (Lys-C). The MALDI-TOF mass spectrometry of the Lys-C digest (Fig. 5, panel A) clearly showed the presence of several peaks (indicated by arrows), which were absent in the control digest mixture of unlabeled GDH protein after the same treatment (panel B). The detailed analysis of labeled peptides is now undergoing in a practical scale for elucidation of the binding site.

In conclusion, a facile and rapid approach for incorporating an acylsulfonamide-based scissile biotin into a binding protein by PAL was developed and allowed for direct purification and isolation of target protein from its mixture. The silver stain of the cleaved product and the MALDI-TOF mass spectrometry of the digest indicated that the method utilizing scissile function successfully gave the labeled product with high purity. All the important purification steps from biotin insertion into the GDH up to mass spectrometry analysis via N-alkylation of acylsulfonamide linker have been performed within a few days. Guarantee to obtain unmodified protein unless modifications have been intentionally introduced into the frame. This methodology including selective alkylation of N- and S-alkylation opens up new route toward the structural elucidation of binding site into the target protein by PAL.

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References and notes

1. (a) Brunner, J. *Annu. Rev. Biochem.* **1993**, *62*, 483; (b) Kotzyba-Hibert, F.; Kapfer, I.; Goeldner, M. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 1296; (c) Dormán, G.; Prestwitch, G. D. *Trends Biotechnol.* **2000**, *18*, 64.
2. (a) Yan, P.; Wang, T.; Newton, G. J.; Knyushko, T. V.; Xiong, Y.; Bigelow, D. J.; Squier, T. C.; Mayer, M. U. *ChemBioChem* **2009**, *10*, 1507; (b) Jahn, O.; Eckart, K.; Brauns, O.; Tezval, H.; Spiess, J. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 12055.
3. (a) Moss, R. A. *Acc. Chem. Res.* **2006**, *39*, 267; (b) Nakashima, H.; Hashimoto, M.; Sadakane, Y.; Tomohiro, T.; Hatanaka, Y. *J. Am. Chem. Soc.* **2006**, *128*, 15092; (c) Blencowe, A.; Hayes, W. *Soft Matter* **2005**, *1*, 178; (d) Dormán, G.; Tomohiro, T.; Hatanaka, Y.; Darvas, F. In *Immunogenomics and Human Disease*; Falus, A., Ed.; John Wiley & Sons, Ltd: Chichester, 2005; pp 69–94. Chapter 4.
4. (a) Hashimoto, M.; Hatanaka, Y. *Eur. J. Org.* **2008**, 2513; (b) Hatanaka, Y.; Hashimoto, H.; Kanaoka, Y. *J. Am. Chem. Soc.* **1998**, *120*, 453.
5. (a) Verhelst, S. H. L.; Forović, M.; Bogyo, M. *Angew. Chem., Int. Ed.* **2007**, *46*, 1284; (b) Zhou, H.; Ranish, J. A.; Watts, J. D.; Aebersold, R. *Nat. Biotechnol.* **2002**, *19*, 512; (c) Fang, K.; Hashimoto, M.; Jockusch, S.; Turo, N. J.; Nakanishi, K. *J. Am. Chem. Soc.* **1998**, *120*, 8543.
6. (a) Speers, A. E.; Cravatt, B. F. *J. Am. Chem. Soc.* **2005**, *127*, 10018; (b) Hashimoto, M.; Okamoto, S.; Nabeta, K.; Hatanaka, Y. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 2447.
7. Bongo, N. B.; Tomohiro, T.; Hatanaka, Y. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 80.
8. Gurd, F. R. N. *Methods Enzymol.* **1967**, *11*, 532.
9. Hatanaka, Y.; Hashimoto, M.; Nishihara, S.; Narimatsu, H.; Kanaoka, Y. *Carbohydrate Res.* **1996**, *294*, 95.
10. Lamos, S. M.; Krusemark, C. J.; McGee, C. J.; Scalf, M.; Smith, L. M.; Belshaw, P. J. *Angew. Chem., Int. Ed.* **2006**, *45*, 4329.