ChemComm

RSC Publishing

COMMUNICATION

View Article Online
View Journal | View Issue

Photoaffinity casting of a coumarin flag for rapid identification of ligand-binding sites within protein†

Cite this: *Chem. Commun.*, 2013, **49**, 1811

Received 30th November 2012,
Accepted 11th January 2013

DOI: 10.1039/c3cc38594a

www.rsc.org/chemcomm

Shota Morimoto,[a] Takenori Tomohiro,*[a] Nobuyuki Maruyama[b] and Yasumaru Hatanaka*[a]

A photo-switchable fluorescent flagging approach has been developed to identify photoaffinity-labeled peptides in target protein. Upon photochemical release of the ligand, the protein was newly modified with a coumarin in place of the previously attached biotin. It allowed us to simplify complex identification processes for labeled sites.

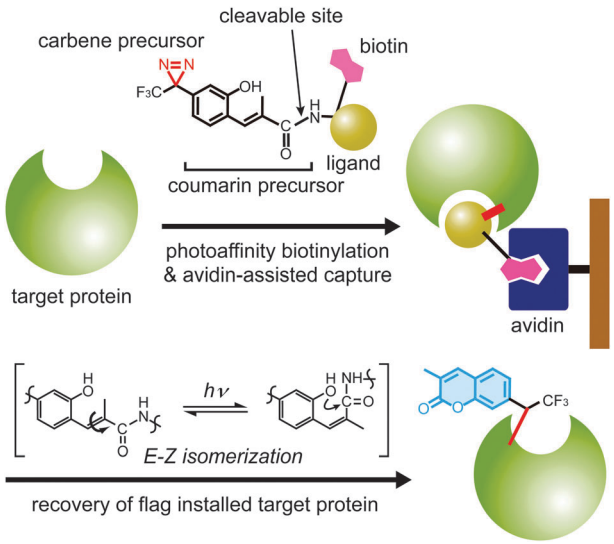
A high throughput photoaffinity-based method has been developed for the identification of ligand-binding domains within proteins. Structural elucidation as well as identification of the target protein is a crucial matter in the field of life sciences for understanding and regulating complex biological processes. Photoaffinity cross-linking with mass spectrometric analysis is a powerful approach to investigate the structure of proteins and their complexes.[1,2] This approach is particularly useful for analyzing proteins that are difficult to analyze by conventional X-ray crystallography and solution-state NMR methods. The cross-linked proteins are subjected to proteolytic digestion and the resulting peptide fragments are analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Despite the simplicity of the cross-linking strategy, identification of cross-linked peptides is often quite troublesome because of the complexity of the reaction mixtures and differences in the physical properties of the peptides.[1] 3-Phenyl-3-trifluoromethyldiazirine has been recognized as an ideal photophore that rapidly creates a stable covalent bond with any spatially closed molecule with minimal diffusion.[3] This is a crucial feature for site-specific labeling at interfaces of physical interactions within proteins. However, regarding the identification of labeled peptide fragments, a trace amount of inevitable adsorption could often greatly interfere with the purification at picomolar concentrations, and assignment of the labeled peptide by LC often fails.

Several strategies have been investigated to facilitate the identification of peptides, *e.g.*, selective purification and detection protocols. Purification has been achieved by introducing a purification tag using a clickable group,[4] a perfluoroalkyl chain,[5] and a biotin moiety.[6] Detection has been performed by using multifunctional cross-linkers bearing isotope-coded,[7] fluorogenic,[8] and cleavable functions.[9] Thus far, diazirine-based biotinylated probes have provided an efficient solution for the one-step purification of labeled proteins using an avidin-immobilized matrix,[6] whereas peptide identification has been greatly affected by some contaminants eluted from the matrix. Several scissile functional groups allow selective recovery of biotinylated products trapped irreversibly on the matrix,[10] while general cleavable approaches inevitably result in the loss of biotin as a sensitive detection tag for further assignment of the labeled peptide. Here, we report a unique and rapid method for identification of a protein domain using a novel photo-cleavable biotin probe. This photoprobe tags a fluorescent label in place of the previously attached biotin tag upon releasing a photoaffinity cross-linked protein.

To complete the identification of the labeled peptide by MS, the photoreactive unit was designed according to essential features based on specificity of labeling, purification efficiency of labeled proteins, selectivity and sensitivity for detection of labeled peptide fragments, and simplicity of handling. The cross-linker employed for this purpose was a newly developed *o*-hydroxycinnamate diazirine compound.[11] This molecule is a small-sized cross-linker in which only one double bond is added to a general phenyldiazirine photophore, which prevents reduction in the affinity between the ligand and the receptor. This compound can perform another photoreaction: *E–Z* photo-isomerization of the cinnamate group, which in turn induces lactonization *via* intermolecular nucleophilic substitution by a hydroxy group at the *ortho* position (Scheme 1).[12] This reaction possesses 2 useful advantages. (1) It releases the large ligand molecule from the cross-linked protein under mild conditions, which enables the selective isolation of protein fragments by using the biotin–avidin system. (2) It concurrently forms a coumarin derivative at the ligand-binding site as a stable fluorescent tag, which enables specific and highly sensitive detection of the labeled products, without the further addition of a

[a] *Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, 2630 Sugitani, Toyama 930-0194, Japan. E-mail: ttomo@pha.u-toyama.ac.jp, yasu@pha.u-toyama.ac.jp; Fax: +81 76-434-5063; Tel: +81 76-434-7516*
[b] *Laboratory of Food Quality Design and Development, Graduate School of Agriculture, Kyoto University, Gokasho, Uji, Kyoto 611-0011, Japan*

† Electronic supplementary information (ESI) available: Materials and methods, inhibition assay, fluorescence spectra, and additional MS and NMR data. See DOI: 10.1039/c3cc38594a



Scheme 1 Tag-switching strategy for the identification of target proteins by double photoreactions of a multifunctional cross-linker.

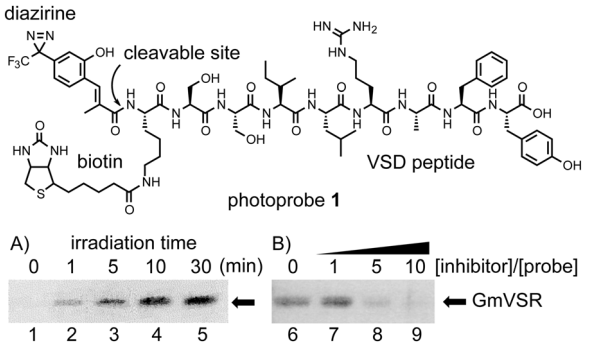


Fig. 1 Structure of photoreactive VSD photoprobe **1** and chemiluminescence detection of the GmVSR cross-linked with photoprobe **1**. Photoreaction was performed under 365 nm light at 0 °C for 0, 1, 5, 10, 30 min (panel A) in the absence (lanes 1–6) or in the presence of an inhibitor (SSILRAFY) in various equivalent mol ratios (lanes 7–9, panel B, samples were irradiated for 10 min).

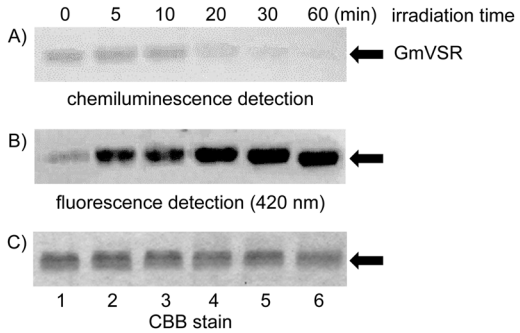


Fig. 2 GmVSR detected by the chemiluminescence method (A) for biotinylated products, fluorescence method (B) for coumarin-labeled products, and CBB staining. Emission from the band was detected through a band filter (420 nm, fwhm 10 nm) under UVA irradiation.

detection tag. Our photophore labeling system also eliminates the necessity of labeling post cleavage and purification. Moreover, compared to a large adduct, a minimally bulky coumarin tag proves to be advantageous for higher resolution mass spectroscopic analysis of the labeled peptide. Briefly, the multifunctional photoprobe can specifically capture and isolate the target protein only by UV-irradiation and easily enable identification of the labeled peptide fragment.

In this study, we applied this concept to the identification of the substrate-binding domain of the vacuolar sorting receptor (VSR) of soybean. During seed maturation, VSR, a membrane protein, transports proteins such as newly synthesized seed storage proteins from the endoplasmic reticulum to the protein storage vacuole *via* a transport vesicle.[13] Recently, a C-terminal peptide of soybean (*Glycine max*) 7S globulin (β-conglycinin) was identified as a vacuolar sorting determinant (VSD).[14,15] Further, we found that the C-terminal sequence (SSILRAFY) of the β-conglycinin α′ subunit is a binding partner for soybean VSR (K_d = 100–200 nM). The photoreactive unit was conjugated to the N-terminus of the peptide *via* biocytin to affix the photoprobe **1** (Fig. 1, see the ESI† for Experimental details). Photoaffinity labeling of a recombinant luminal soybean VSR (GmVSR, 1 μM) by photoprobe **1** (1 μM) was carried out with 360 nm light (a 30 W black light lamp) in a buffered solution containing 20 mM Hepes (pH 7.0), 150 mM NaCl, 1 mM $CaCl_2$, 0.4% chaps, and 0.02% NaN_3 at 0 °C. Photoproducts were then subjected to 10% SDS–PAGE. The labeled products were detected by chemiluminescence methods using horseradish peroxidase (HRP)-conjugated avidin after blotting onto a PVDF membrane because the products were biotinylated. The emission intensity of the band around 55 kDa increased depending on the irradiation time (Fig. 1A) and clearly decreased in the presence of SSILRAFY, which served as an inhibitor (Fig. 1B). In addition, competitive binding assays were performed with other VSD analogs, in which 1 or 2 amino acid residues were substituted with glycine. These peptides had reduced affinity to GmVSR, which exhibited similar inhibitory patterns compared to previous results obtained from sorting experiments of green fluorescence protein fused with β-conglycinin C-terminal peptides (see the ESI†).[15] These results indicate that specific labeling of photoprobe **1** at the VSD-binding domain was achieved. The yield of cross-linking was about 5%, which was determined by comparison with the emission intensity of biotinylated BSA.

The second photoreaction was subsequently carried out with 315 nm light (15 W) at 25 °C. The emission intensity of the ligand-cross-linked GmVSR band decreased with the irradiation time in response to the release of a biotin group (Fig. 2A). At the same time, the intensity of fluorescence due to the coumarin fluorophore increased at the same position (Fig. 2B). The result of Coomassie Brilliant Blue (CBB) staining indicated that photodegradation of GmVSR did not occur during the irradiation (Fig. 2C). The intramolecular nucleophilic substitution by a hydroxy group proceeded efficiently at 25 °C to create a coumarin derivative. While some of the cross-linked GmVSR allowed coumarin formation during the denaturing process at room temperature (lane 1, Fig. 2B), the 2 reactions of cross-linking and cleavage were well regulated by changing irradiation wavelength and reaction temperature.

We then applied this fluorogenic photo-cleavage reaction for purification of the labeled protein. After removal of the

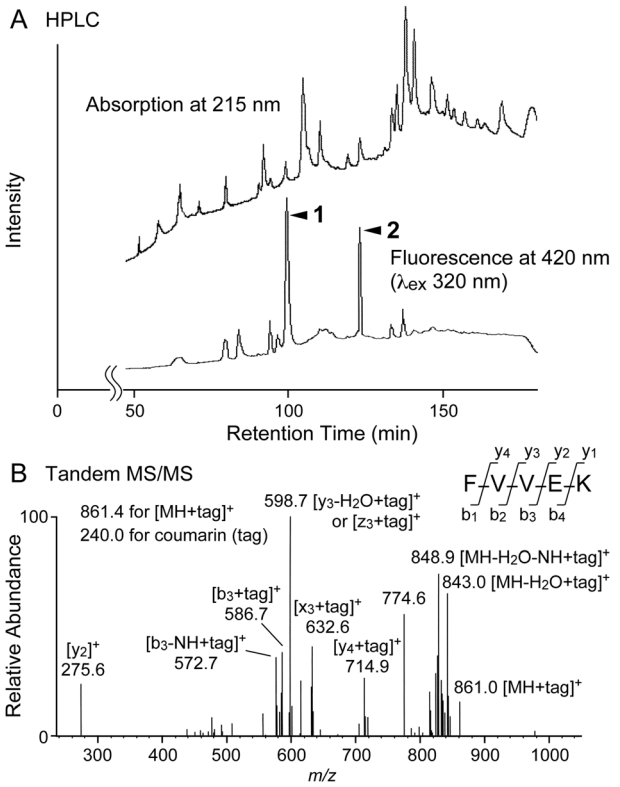


Fig. 3 (A) Reverse-phase HPLC profiles of the digested products showing lysyl endopeptidase detected by absorption at 215 nm (upper) and fluorescence at 420 nm (λ_{ex} = 320 nm). (B) Tandem MS/MS spectrum of the $[FVVEK + H]^+$ ion (peak 1, m/z 861.50 in Fig. S3B, ESI†).

non-cross-linked probe by dialysis, the cross-linked products were subjected to an avidin-immobilized agarose gel. After washing with 0.2% SDS-containing PBS solution several times, the gel was exposed to 315 nm light for one hour at 25 °C. Pure, labeled GmVSR was recovered from the solid matrix in 60% yield, which was estimated using CBB staining. The labeled product showed fluorescent emission due to the corresponding coumarin. This reaction was mild, selective, and efficient, and yielded almost pure protein without contamination with other adsorbed materials.

After digestion of the labeled GmVSR with lysyl endopeptidase, the resulting peptides were separated by reverse-phase HPLC. As shown in the HPLC profiles (Fig. 3A), and compared to the result detected by UV absorption at 215 nm, there were a limited number of peaks, which were detected easily with high sensitivity using emission at 420 nm (λ_{ex} = 320 nm). These peaks appeared identical to the labeled peptides because of the presence of coumarin. This is one of the most beneficial things in this method from a standpoint of skipping the hampered assignment processes. Two major peaks indicated by arrows were identified by ESI-MS as the coumarin-modified FVVEK ($\Delta m/z$ = 240.0 u) (861.4 for MH^+ + coumarin, found 861.5) and VWNAQK (985.4 for MH^+ + coumarin, found 985.0). The results of MS identification from labeling were reproducible. These peptides are derived from the N-terminal region at the 2nd to 6th residues and 85th to 90th residues, respectively. The tandem mass spectrum acquired for the single-charged $[FVVEK + H]^+$ ion display b_n + 240 u, y_n + 240 u series, containing a coumarin modification (Fig. 3B). There are y_3 + 240 u, y_4 + 240 u ions, and unmodified y_2 ions, as well as b_3 + 240 u ions. The spectrum demonstrated that the coumarin modification occurred on the valine residue at the 3rd position from the N-terminus of the fragment.

In conclusion, the photochemical coumarin tagging method allowed us to identify the labeled peptides rapidly. The requisite amount of protein for analysis is in the 10 micrograms range. In addition, this approach can identify multiple peptide components of a binding domain that could not be characterized by conventional photolabeling approaches. The label-switching strategy coupled with high-selective purification should give a great advantage for identification of the target proteins of low abundance, which is an inherent problem in techniques such as shotgun proteomics.[16]

This research was supported by Grants-in-Aid for Scientific Research (20390032, 21310138) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Notes and references

1 (*a*) B. Domon and R. Aebersold, *Science*, 2006, **312**, 212; (*b*) A. Sinz, *Mass Spectrom. Rev.*, 2006, **25**, 663; (*c*) K. J. Pacholarz, R. A. Garlish, R. J. Taylor and P. E. Barran, *Chem. Soc. Rev.*, 2012, **41**, 4335; (*d*) T. Lenz, J. J. Fischer and M. Dreger, *J. Proteomics*, 2011, **75**, 100.
2 M. A. Trakselis, S. C. Alley and F. T. Ishmael, *Bioconjugate Chem.*, 2005, **16**, 741.
3 (*a*) J. Brunner, *Annu. Rev. Biochem.*, 1993, **62**, 483; (*b*) A. Blencowe and W. Hayes, *Soft Matter*, 2005, **1**, 178; (*c*) J. Das, *Chem. Rev.*, 2011, **111**, 4405; (*d*) J. Wang, J. Kubicki, H. Peng and M. S. Platz, *J. Am. Chem. Soc.*, 2008, **130**, 6604.
4 (*a*) C. X. Song and C. He, *Acc. Chem. Res.*, 2011, **44**, 709; (*b*) C. H. Sohn, H. D. Agnew, J. E. Lee, M. J. Sweredoski, R. L. J. Graham, G. T. Smith, S. Hess, G. Czerwieniec, J. A. Loo, J. R. Heath, R. J. Deshaies and J. L. Beauchamp, *Anal. Chem.*, 2012, **84**, 2662; (*c*) A. L. MacKinnon and J. Taunton, *Curr. Protoc. Chem. Biol.*, 2009, **1**, 55; (*d*) M. Hashimoto and Y. Hatanaka, *Chem. Pharm. Bull.*, 2005, **53**, 1510.
5 Z. Song, W. Huang and Q. Zhang, *Chem. Commun.*, 2012, **48**, 3339.
6 (*a*) T. Tomohiro, M. Hashimoto and Y. Hatanaka, *Chem. Rec.*, 2005, **5**, 385; (*b*) Y. Hatanaka, M. Hashimoto and Y. Kanaoka, *Bioorg. Med. Chem.*, 1994, **2**, 1367; (*c*) Y. Luo, C. Blex, O. Baessler, M. Glinski, M. Dreger, M. Sefkow and H. Köster, *Mol. Cell. Proteomics*, 2009, **8**, 2843.
7 (*a*) K. K. Palaniappan, A. A. Pitcher, B. P. Smart, D. R. Spiciarich, A. T. Iavarone and C. R. Bertozzi, *ACS Chem. Biol.*, 2011, **6**, 829; (*b*) M. Hashimoto and Y. Hatanaka, *Chem. Pharm. Bull.*, 2004, **52**, 1385.
8 (*a*) E. W. Chan, S. Chattopadhaya, R. C. Panicker, X. Huang and S. Q. Yao, *J. Am. Chem. Soc.*, 2004, **126**, 14435; (*b*) T. Kuroda, K. Suenaga, A. Sakakura, T. Handa K. Okamoto and H. Kigoshi, *Bioconjugate Chem.*, 2006, **17**, 524.
9 (*a*) Y. Chen, Y. W. Ebright and R. H. Ebright, *Science*, 1994, **265**, 90; (*b*) J. J. Park, Y. Sadakane, K. Masuda, T. Tomohiro, T. Nakano and Y. Hatanaka, *ChemBioChem*, 2005, **6**, 814; (*c*) A. F. Gomes and F. C. J. Gozzo, *J. Mass Spectrom.*, 2010, **45**, 892.
10 G. Leriche, L. Chisholm and A. Wagner, *Bioorg. Med. Chem.*, 2012, **20**, 571, and references therein.
11 T. Tomohiro, K. Kato, S. Masuda, H. Kishi and Y. Hatanaka, *Bioconjugate Chem.*, 2011, **22**, 315.
12 A. D. Turner, S. V. Pizzo, G. Rozakis and N. A. Porter, *J. Am. Chem. Soc.*, 1988, **110**, 244.
13 (*a*) T. Shimada, K. Fuji, K. Tamura, M. Kondo, M. Nishimura and I. Hara-Nishimura, *Proc. Natl. Acad. Sci. U. S. A.*, 2003, **100**, 16095; (*b*) A. Vitale and N. V. Raikhel, *Trends Plant Sci.*, 1999, **10**, 316.
14 K. Nishizawa, N. Maruyama and S. Utsumi, *Plant Mol. Biol.*, 2006, **62**, 111.
15 K. Nishizawa, N. Maruyama, R. Satoh, Y. Fuchikami, T. Higasa and S. Utsumi, *Plant J.*, 2003, **34**, 647.
16 P. Picotti, R. Aebersold and B. Domon, *Mol. Cell. Proteomics*, 2007, **6**, 1589.