Yan Wang[1,2]
Mingming Dong[1,3]
Mingliang Ye[1,2]

[1]CAS Key Laboratory of Separation Sciences for Analytical Chemistry, University of Chinese Academy of Sciences, Beijing, P. R. China
[2]National Chromatographic R&A Center, Dalian Institute of Chemical Physics, Chinese Academy of Sciences (CAS), Dalian, P. R. China
[3]Dalian Ocean University, Dalian, P. R. China

Received December 26, 2018
Revised February 9, 2019
Accepted February 10, 2019

Short Communication

A chemoenzymatic approach enables the site-specific conjugation of recombinant proteins

Many biotechniques including protein microarray, drug screening, biosensors rely on the immobilization of recombinant proteins on the solid supports. It is well known that random orientation of the immobilized proteins could impair their biologic functions. Thus, it is very important to develop new site-specific immobilization approach. In this study, we presented a chemoenzymatic approach for site-specific conjugation of recombinant proteins onto solid support. In this strategy, the affinity tag on recombinant protein was enzymatically cleaved to expose the N-terminal serine, which was oxidized to carry an aldehyde group and was then covalently coupled to hydrazide resin through hydrazone ligation. As this approach takes advantage of the most frequently used TEV protease, it requires no further sequence design on recombinant protein. This method was validated by site specific coupling of a synthetic peptide and a recombinant protein onto solid supports. It was found that the site specific immobilized SH2 domain is functional and could be used to enrich tyrosine phosphorylated peptides.

Keywords:
Biorthogonal chemistry / Chemoenzymatic approach / Site-specific conjugation / Tyrosine phosphorylation
DOI 10.1002/elps.201800528

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Many biotechniques including protein microarray, drug screening, biosensors rely on the immobilization of active biomolecules, in most cases recombinant proteins, on the solid supports [1]. The functional groups on proteins such as primary amino groups on N-termini and lysine side chains are often used for protein immobilization. However, protein function could be impaired because of random orientation of the immobilized proteins [2, 3]. Controlling orientation and coupling chemistry of proteins on surfaces is critical for optimal functioning. Recombinant proteins could be site specifically immobilized onto solid supports by exploiting the affinity interactions between biomolecules. For example, His-tag containing proteins could be easily immobilized onto the solid supports functionalized with metal ions in a site-specific way. However, such noncovalent immobilization is not stable and not suitable for long term usage. A few site-specific, covalent immobilization approaches are also available. Recombinant proteins could be immobilized by the introduction of an unnatural amino acid with orthogonal chemical reactivity to the position to be immobilized [4, 5]. However, such methods need strong expertise on molecular biology operation and protein engineering. Another type of method is the enzyme mediated approach. For example, Sortase A mediated ligation enabled covalent immobilization of recombinant proteins onto the solid supports through the ligation reaction between a substrate motif (LPXTG) and oligoglycine nucleophiles. Such chemoenzymatic approaches are easy to perform and have gained popular recently [6–8]. However, the relatively low catalytic efficiency and reversibility of the ligation reaction limit its potential applications [9]. In this study, we presented a chemoenzymatic approach to enable covalent immobilization of recombinant proteins on the solid supports through its N-termini.

Affinity tags on recombinant proteins enable their purification from total cell lysate. However, presence of an affinity tag may lead to the change of protein conformation and its function. To avoid this problem, the tag could be cleaved after purification. The tobacco etch virus (TEV) protease is a

Correspondence: Professor Mingliang Ye, CAS Key Laboratory of Separation Sciences for Analytical Chemistry, University of Chinese Academy of Sciences, Beijing 100049, P. R. China
Fax: +86-411-84379620
E-mail: mingliang@dicp.ac.cn

Abbreviations: pTyr, phosphorylated tyrosine; **TEV**, tobacco etch virus

Color online: See the article online to view Figs. 1 and 3 in color.

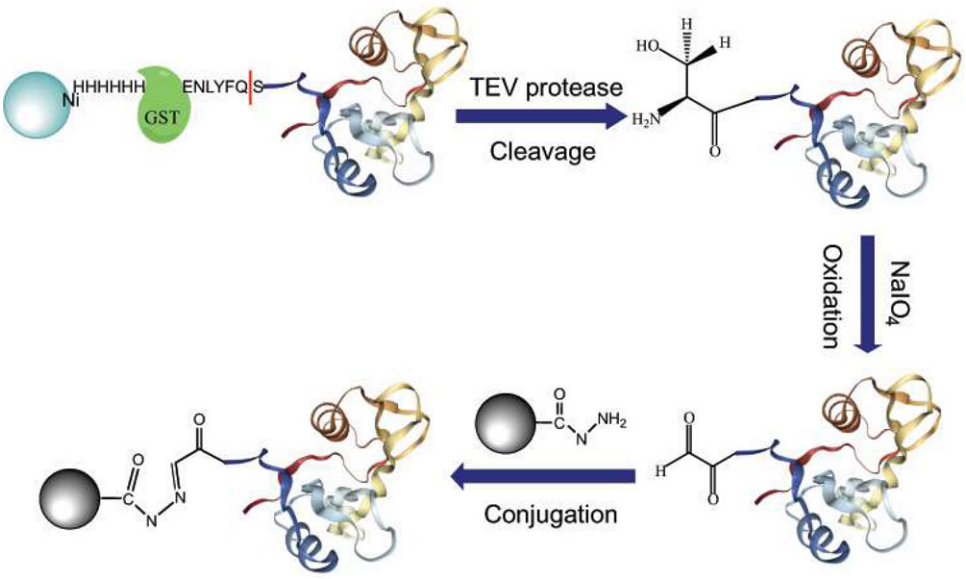

Figure 1. Site-specific conjugation of recombinant proteins by enzymatically release of affinity tag followed by mild oxidation of N-terminal serine, and hydrazone ligation with hydrazide resin.

popular tag cleavage enzyme, which is a highly specific proteolytic enzyme that recognizes the seven-amino-acid sequence EXXYXQ(G/S) (where X can be any amino acid) and cleavages between glutamine and glycine or serine. The chemoenzymatic approach presented in this study allows the removal of affinity tag and, in the meantime, enables the site-specific, covalent immobilization of recombinant proteins on the solid supports. As shown in Fig. 1, the TEV protease recognizing tag EXXYXQS is placed between the affinity tag and the sequence for the protein of interest. After the protein is expressed, it could be purified by the affinity tag. And then the TEV protease is used to cleave the seven-amino-acid sequence which allowed the removal of the affinity tag. Thus, the resulting recombinant protein has an N-terminal Ser. It is known that the 1, 2-amino alcohol structure at N-terminal serine can be oxidized by sodium periodate into the aldehyde group [10, 11], which is similar with the oxidation of cis-diol structure in glycoprotein / peptides for glycoproteomics analysis [12, 13]. This oxidation condition is mild and does not result in obvious side reaction. This method can be even used to oxidize glycans on the cell surface of living cells for the study of cell surface proteome [14]. After the oxidation, an aldehyde group is generated on the N-termini of the recombinant protein. When this protein is incubated with the solid phase supports functionalized with hydrazine group, it will be conjugated to the support surface via its N-termini. In this way, site-specific, covalent conjugation of recombinant proteins could be achieved.

This method was first validated by using a synthetic peptide (P1, ENLYFQSKETAAAKFER) containing the seven-amino-acid sequence recognized by TEV protease. This peptide has a molecular weight of 2032.24 Da (Fig. 2A). After incubating the peptide with TEV protease, two peptide fragments with m/z of 812.88 (P2, ENLYFQ) and 1237.38 (P3, SKETAAAKFER) were observed (Fig. 2B), indicating the peptide bond between the residues of Q and S was cleaved as expected. The original peptide was not observed, indicating the enzymatically digestion was quite complete. After this step, a peptide with N-terminal serine (P3) was generated. We then investigated if P3 could be oxidized to carry an aldehyde group on the N-termini. For comparison, the original peptide (P1) and the product (P3) were oxidized with sodium periodate. The decreasing of 31.06 Da of oxidized peptide (P4, α-oxo-aldehyde-KETAAAKFER) corresponding to the formation of an aldehyde group was only observed on P3. Since P3 is a C-terminal fragment of P1, all the side chains of P3 are also present on P1. The only difference is that P3 has the 1, 2-amino alcohol structure at N-termini due to the presence of terminal serine. Thus, the aldehyde group must be formed on the N-termini of P3. It can be seen from Fig. 2C that P3 was not observed in the spectra after oxidation indicating the oxidation was complete. This experiment indicated that TEV protease digestion followed with sodium periodate oxidation can cleave the peptide at designed position and enable the formation of the aldehyde group on the N-termini of the yielded C-terminal fragment which makes the site-specific coupling of the fragment to hydrazide beads possible.

This method was further validated by using a special designed recombinant protein. As shown in Fig. 1, it has two affinity tags, i.e. His tag and GST tag, on the N-termini and a mutant SH2 domain on the C-termini. In the between, there is the seven-amino-acid sequence recognized by TEV protease. To express this protein, E. coli with pETM-30 vector containing the protein sequence were cultured. After lysed with sonification, the recombinant protein was captured by Ni-Sepharose beads via the His tag and then eluted by 500 mM imidazole. The resulting solution was desalted by Sephadex column and then subjected to TEV protease digestion. We investigated cleavage efficiency with various enzyme to substrate ratios and digestion times (Supporting Information Fig. S2a). It was found that the recombinant protein (MW 41 kDa) could be completed cleaved at ratio of 50:1(w/w,

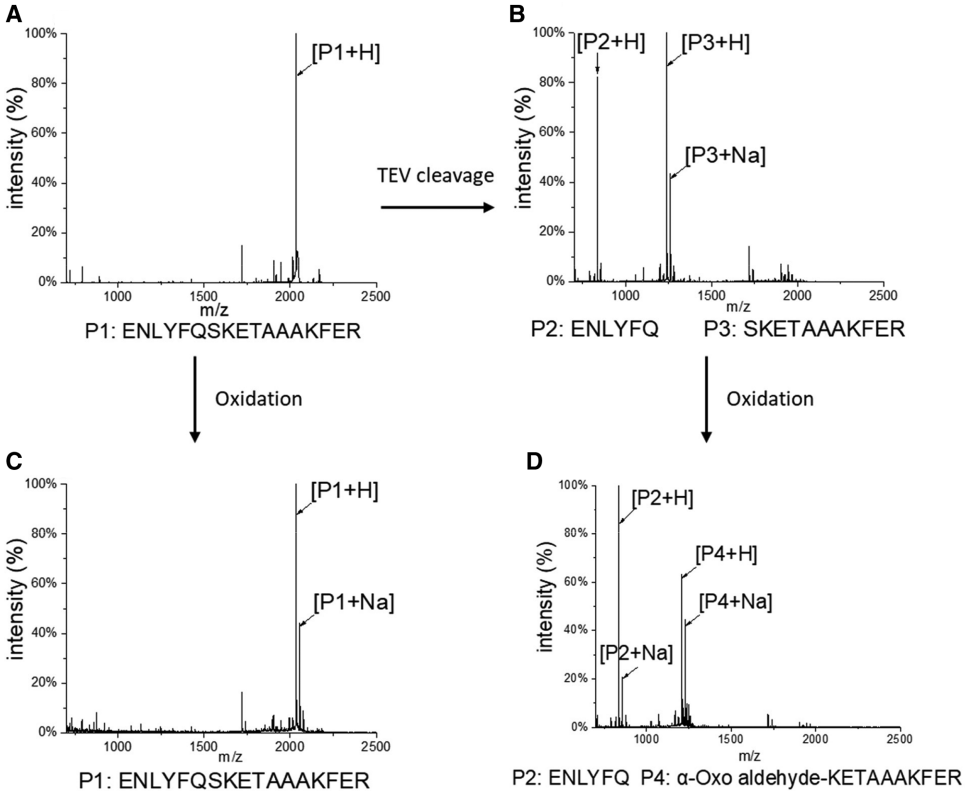

Figure 2. Validation of the approach by using a synthetic peptide (A) MS spectra of the synthetic peptide; (B) cleavage with TEV protease; (C) uncleaved peptide was oxidized by sodium periodate; (D) cleaved peptide was oxidized by sodium periodate.

substrate: enzyme) for 3 h (Supporting Information Fig. S2b, lane 3). After cleavage, the SH2 domain protein was purified by size exclusion chromatography and oxidized by sodium periodate. It was found that the mobility of oxidized SH2 domain protein in the gel was decreased compared with the unoxidized form which could be partially attributed to the hydrophobicity change (Supporting Information Fig. S2b, lane 4) [15]. We then tested if the oxidized proteins could be coupled onto hydrazide resins. The oxidized proteins were incubated with hydrazide resins and the remaining solution was analyzed by SDS-PAGE (Supporting Information Fig. S2b, lane 5). This protein band was not observed, which indicated the high coupling efficiency.

Above example indicated that the affinity tags could be efficiently cleaved and the SH2 domain protein could be efficiently coupled onto the hydrazide resins. However, the procedure is quite tedious. We then investigate if we can skip the desalting and chromatographyic purification steps by performing on beads cleavage. Firstly, the Ni-Sepharose beads were saturated with the recombinant His/GST-tagged protein. On-beads cleavage was processed by adding TEV protease to beads slurry and rotated in an end-to-end manner. The flowthrough containing N-terminal serine residual SH2 domain was directly oxidized by sodium periodate and analyzed by SDS-PAGE. The results were found to be consistent with the theoretical molecular weight that the fusion protein GST-SH2 is about 41k Da while the digested and the oxidized SH2 are about 15k Da (Supporting Information Fig. S1). The coupling efficiency was evaluated by both SDS-PAGE analysis and the concentration of desalted SH2 before and after conjugation. When staining by Coomassie blue, the remnant SH2 after coupling was hardly detectable as the in-solution method (Supporting Information Figs. S1 and S2b) while the coupling efficiency was determined to be about 70% according to the concentration changes after conjugation.

After the protein was site-specific coupled to solid phase supports, another key issue is whether the protein preserves its bioactivity. The SH2 domain is the reader to recognize the tyrosine phosphorylation on proteins. The mutant SH2 domain used in this study was obtained by introducing three mutations into the phosphorylated tyrosine (pTyr)-binding pocket of the wild type Src SH2 domain, which significantly enhanced its affinity to tyrosine phosphorylation [16, 17]. For this reason, this domain is termed as SH2 superbinder. To evaluate its bioactivity, the immobilized SH2 superbinder was applied to capture the pTyr peptides from complex peptide mixture (Fig. 3). The lysate of Jurkat cells was digested by trypsin and the phosphopeptides in the digest were enriched with immobilized titanium (IV) ion affinity chromatography (Ti^{4+}-IMAC) [18]. The obtained phosphopeptides were subjected to LC-MS/MS analysis, which resulted in identification of 5152 phosphopeptides. Among these phosphopeptides, only 8.25% (425) had pTyr sites. Clearly majority of phosphopeptides in this mixture were pSer/pThr. This phosphopeptide mixture was incubated with the SH2 superbinder conjugated-hydrazide beads, the captured peptides were eluted by formic acid

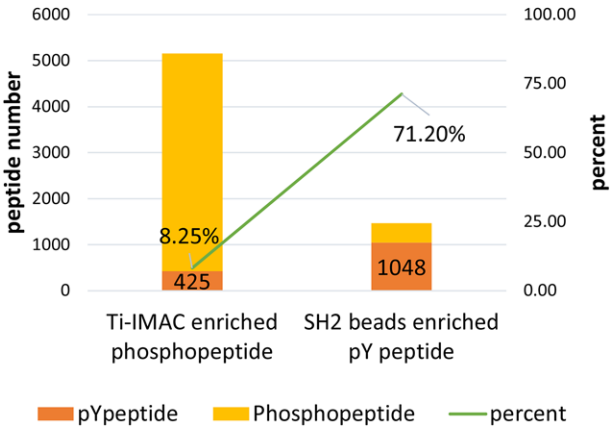

Figure 3. Site specific conjugated SH2 domain remains functional and allows capture of pTyr peptides from phosphopeptides mixture.

and analyzed by LC-MS/MS. This leaded to identification of 1472 phosphopeptides. Among them, 71.2% (1048) were pTyr phosphopeptides. After the capture by the immobilized SH2 superbinder, the percentage of pTyr peptides increased from 8.25% to 71.2% and the number of pTyr peptides increased from 425 to 1048 (Fig. 3). These results clearly indicated that the conjugated SH2 domain preserved its activity of recognition to pTyr and could be used for purification of pTyr peptides.

Here we presented a chemoenzyamtic strategy to achieve the site-specific conjugation of recombinant proteins onto solid supports. In this strategy, the affinity tag on recombinant proteins was enzymatically cleaved to expose the N-terminal serine, which was then oxidized to carry an aldehyde group and was covalently coupled to hydrazide resin through hydrazone ligation. It was found that the site specific immobilized SH2 domain is functional and could be used to enrich tyrosine phosphorylated peptides. The on-beads cleavage followed with oxidation reduced the steps of protein elution from affinity resin and the following desalting, purification steps which simplified the immobilization procedure. Because this approach exploited the most frequently used TEV protease to cleave the affinity tag, it requires no further sequence design on recombinant proteins. Compared to other biorthogonal chemistry-based methods, this N-terminal specific conjugation approach is cost effective and could be easily scaled up. We expect this method has broad application in fields using immobilized proteins such as biochips, biosensors etc. However, it should be mentioned that the uniform terminal specific conjugation of recombinant proteins onto solid supports cannot be achieved if the proteins are glycosylated. This is because the attached glycans could also be oxidized by periodate and be conjugated onto the solid beads.

Financial support is gratefully acknowledged from the National Key R&D Program of China (2016YFA0501402, 2017YFA0505004), the National Natural Science Foundation of China (21535008, 21605140). M.Y. is a recipient of the National Nature Science Fund of China for Distinguished Young Scholars (21525524).

The authors have declared no conflict of interest.

References

[1] Steen Redeker, E., Ta, D. T., Cortens, D., Billen, B., Guedens, W., Adriaensens, P., *Bioconjug. Chem.* 2013, *24*, 1761–1777.

[2] van Vught, R., Pieters, R. J., Breukink, E., *Comput. Struct. Biotechnol. J.* 2014, *9*, e201402001.

[3] MacDonald, J. I., Munch, H. K., Moore, T., Francis, M. B., *Nat. Chem. Biol.* 2015, *11*, 326–331.

[4] Greiss, S., Chin, J. W., *J. Am. Chem. Soc.* 2011, *133*, 14196–14199.

[5] Kim, C. H., Axup, J. Y., Dubrovska, A., Kazane, S. A., Hutchins, B. A., Wold, E. D., Smider, V. V., Schultz, P. G., *J. Am. Chem. Soc.* 2012, *134*, 9918–9921.

[6] Lotze, J., Reinhardt, U., Seitz, O., Beck-Sickinger, A. G., *Mol. Biosyst.* 2016, *12*, 1731–1745.

[7] Kuropka, B., Royla, N., Freund, C., Krause, E., *Proteomics* 2015, *15*, 1230–1234.

[8] Wang, H. H., Altun, B., Nwe, K., Tsourkas, A., *Angew. Chem. Int. Ed.* 2017, *56*, 5349–5352.

[9] Schmohl, L., Schwarzer, D., *Curr. Opin. Chem. Biol.* 2014, *22*, 122–128.

[10] Geoghegan, K. F., Stroh, J. G., *Bioconj. Chem.* 1992, *3*, 138–146.

[11] Yao, Y., Huang, J., Cheng, K., Pan, Y., Qin, H., Ye, M., Zou, H., *Anal. Chem.* 2015, *87*, 11353–11360.

[12] Zhang, H., Li, X.-j., Martin, D. B., Aebersold, R., *Nat. Biotechnol.* 2003, *21*, 660.

[13] Nilsson, J., Rüetschi, U., Halim, A., Hesse, C., Carlsohn, E., Brinkmalm, G., Larson, G., *Nat. Meth.* 2009, *6*, 809.

[14] Wollscheid, B., Bausch-Fluck, D., Henderson, C., O'Brien, R., Bibel, M., Schiess, R., Aebersold, R., Watts, J. D., *Nat. Biotechnol.* 2009, *27*, 378.

[15] Shirai, A., Matsuyama, A., Yashiroda, Y., Hashimoto, A., Kawamura, Y., Arai, R., Komatsu, Y., Horinouchi, S., Yoshida, M., *J. Biol. Chem.* 2008, *283*, 10745–10752.

[16] Bian, Y., Li, L., Dong, M., Liu, X., Kaneko, T., Cheng, K., Liu, H., Voss, C., Cao, X., Wang, Y., Litchfield, D., Ye, M., Li, S. S., Zou, H., *Nat. Chem. Biol.* 2016, *12*, 959–966.

[17] Kaneko, T., Huang, H., Cao, X., Li, X., Li, C., Voss, C., Sidhu, S. S., Li, S. S., *Sci. Signal.* 2012, *5*, ra68.

[18] Zhou, H., Ye, M., Dong, J., Corradini, E., Cristobal, A., Heck, A. J. R., Zou, H., Mohammed, S., *Nat. Protoc.* 2013, *8*, 461.

