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Development of a Simple Method for Protein Conjugation by Copper-Free Click Reaction and Its Application to Antibody-Free Western Blot Analysis

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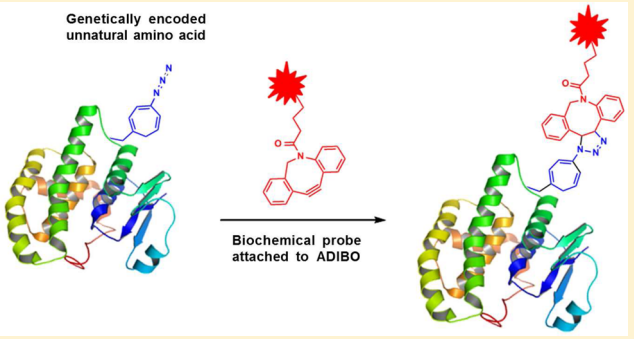
Supporting Information

ABSTRACT: There are currently many methods available for labeling proteins in order to study their structure and function. However, the utility of these methods is hampered by low efficiency, slow reaction rates, nonbiocompatible reaction conditions, large-sized labeling groups, and the requirement of specific side chains such as cysteine or lysine. In this study, a simple and efficient method for protein labeling was developed, in which an azide-containing amino acid was introduced into a protein and conjugated to a labeling reagent by strain-promoted azide−alkyne cycloaddition (SPAAC). This method allowed us to label proteins by simply mixing a protein and a labeling reagent in physiological conditions with a labeling yield of approximately 80% in 120 min. In addition, the specificity of SPAAC made it possible to analyze the expression level of a protein quantitatively by simple mixing and SDS-PAGE analysis with no need for antibodies or multistep incubations. Because the genetic incorporation of the azide-containing amino acid can be generally applied to any protein and the SPAAC reaction is highly specific, this method should prove useful for labeling and analyzing proteins.



INTRODUCTION

A number of chemical tools have been developed labeling proteins with biochemical probes in order to study protein structure and function. One such method is the chemical modification of proteins in which a biochemical probe is covalently attached to cysteines or lysines by alkylation or amide bond formation reactions.[1,2] Although chemical modification is useful and has been applied to many different proteins and probes, it is dependent on the reactivity of specific amino acid side chains, and therefore, the intrinsic selectivity and the overall efficiency of this approach is limited. Alternative methods include labeling proteins with peptide/protein tags such as fluorescent proteins,[3] SNAP-tag,[4] HaloTag,[5] and tetracysteine.[6] Although these tagging methods are particularly useful for in vivo applications, most of these tags can perturb the structure and function of proteins because of their large size. Moreover, the restriction of tag locations to proteins' N- or C-terminals also limits their application.

In order to overcome these limitations, unnatural amino acids with bio-orthogonal reactivity have been incorporated into proteins by using a recently developed genetic incorporation method.[7−9] The major advantage of this method is that it allows for site-specific conjugation of proteins with a biochemical probe. In addition, the method is technically simple and applicable to any protein. The bio-orthogonal functional groups incorporated into proteins by the genetic method include ketones,[10] azides,[11,12] alkynes,[13] alkenes,[14] aryl halides,[15] and aryl boronates.[16] Although site-specific incorporation of these functional groups into proteins is quite useful, the method's applicability is limited by harsh reaction conditions and the slow reaction rate of conjugation reactions. Imine formation by ketones and alkoxyamines requires acidic conditions (pH 4−5),[17] and the click reaction involving azides and alkynes requires biologically incompatible copper(I) catalysts.[18] Recently, copper-free click reactions with cyclooctyne,[19] cyanobenzothiazole condensation with 1,2-aminothiol,[20] and tetrazine-based cycloaddition to norbornene-containing proteins[21] have introduced significant improvements, but continue to have limitations such as low conjugation yields, slow reaction rates, the necessity of an extra step and challenges in the synthesis of labeling compounds. There is, therefore, a need for improved methods for efficiently labeling proteins by simply mixing the protein of interest with a labeling reagent under physiologically friendly conditions. Here, we

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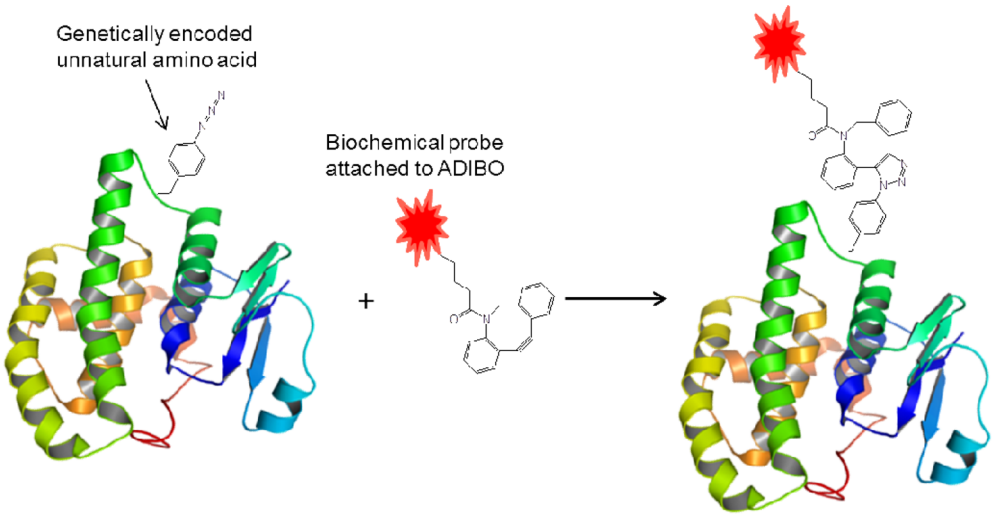

Figure 1. Protein conjugation with a genetically encoded azide-containing protein. An azide-containing amino acid is genetically incorporated into a protein and the azido group reacts with a conjugation reagent containing dibenzocyclooctyne.

have developed a simple and efficient method of introducing a biochemical probe into proteins by strain-promoted azide-akyne cycloaddition (SPAAC) with a genetically encoded azide-containing amino acid.

EXPERIMENTAL PROCEDURES

General. All chemicals and DNA oligomers were obtained from commercial sources and used without further purification. Fluorescence images for SDS-PAGE analysis were obtained using the Typhoon 9210 variable mode imager.

Synthesis of Cy5.5-Linked Aza-dibenzocyclooctyne (Cy5.5-ADIBO). Diisopropylethylamine (0.45 mg, 3.6 μmol) was added to a mix of aza-dibenzocyclooctynyl amine derivative[22,23] (ADIBO-NH_2, 4.0 mg, 13.8 μmol) and Cy5.5 NHS-ester (2.0 mg, 1.8 μmol) in anhydrous DMF (300 μL), and the reaction mixture was stirred at room temperature for 1 h. The crude product was purified by RP-HPLC (C_{18} silica gel, 10 μm, 10 × 250 mm; 10 mM aqueous NH_4HCO_3/acetonitrile = 70:30 (v/v); 254 nm; 2 mL/min) to obtain Cy5.5-ADIBO (2.0 mg, 93%). The Cy5.5-ADIBO product was confirmed by MALDI-TOF-MS: m/z 1211.08 for [M-3Na] ($C_{62}H_{59}N_4O_{14}S_4$ calculated molecular weight [MW] 1211.29). Retention time = 16.4 min.

Expression and Purification of the Mutant GST Containing AZF. A wild-type GST gene was amplified from a commercial vector containing the gene and inserted between the NcoI and *Kpn*I sites of pBAD/Myc-His (Invitrogen) to generate pBAD-GST. Site-directed mutagenesis was used to introduce an amber mutation (F47TAG) into the GST gene. The plasmid containing the amber mutation (pBAD-GST-F47TAG) was cotransformed with pEvol-AZF[24] into *E. coli* DH10B. Cells were amplified in lysogeny broth (LB) supplemented with ampicillin (100 μg/mL) and chloramphenicol (35 μg/mL). Starter culture (2.5 mL) was used to inoculate 100 mL LB supplemented with ampicillin (100 μg/mL), chloramphenicol (35 μg/mL), and 1 mM AZF at 37 °C; and the expression was induced at OD 0.8 (550 nm) by adding L-arabinose (0.2%, final concentration). Cells were grown at 37 °C overnight, harvested by centrifugation, and frozen at −80 °C. Glutathione resin purification was performed according to the manufacturer's protocol (Clontech Laboratories, Inc.). Protein concentrations were calculated by measuring absorbance at 280 nm and by using calculated extinction coefficients (4.3×10^4 cm^{-1} M^{-1}) (http://www.biomol.net/en/tools/proteinextinction.htm).

Conjugation Reactions with Purified GST. The conjugation reaction was performed in 10 mM phosphate buffer (pH 7.0) containing 100 mM NaCl by adding the GST mutant (GST-F47AZF) (20 μM, final concentration) and Cy5.5-ADIBO (200 μM, final concentration) at room temperature. The reaction was quenched by adding excess AZF and the mixture was directly analyzed by SDS-PAGE. Fluorescence images were taken using the Typhoon 9210 variable mode imager and the gel was stained with Coomassie Brilliant Blue R-250.

Conjugation Reactions with Crude Cell Extract. Cells from GST-F47AZF-expressing bacterial cell cultures (50 mL) were harvested by centrifugation and frozen at −80 °C. The frozen cell pellets were thawed on ice, resuspended in 10 mM phosphate buffer (pH 7.0, 5 mL) containing 100 mM NaCl, and sonicated. Alternatively, the thawed cells were resuspended in BugBuster (Novagen) containing 10 mM phosphate buffer (pH 7.0), 100 mM NaCl, and benzonase (125 U/ml) (cat. No. 70746−3, Novagen) and incubated for 30 min at room temperature. Removal of cell debris by centrifugation (12 000 rpm) afforded crude cell extracts. The conjugation reaction was performed by adding Cy5.5-ADIBO (200 μM, final concentration) to the cell extract (10 μL) and incubating the reaction mixture for 1 h at 37 °C. The reaction was quenched by adding excess AZF and the mixture was directly analyzed by SDS-PAGE.

Conjugation Reactions for Analysis of Protein Expression Levels (Antibody-Free Western Blot Analysis). Cells were grown using the method described above, with different concentrations of L-arabinose. For lysate preparation, conjugation reactions, and SDS-PAGE analysis, the same methods described in the previous section were used.

RESULTS AND DISCUSSION

Strain-Promoted Azide−Alkyne Cycloaddition (SPAAC). It has been well-known that SPAAC with dibenzocyclooctyne derivatives is ideal for bioconjugation since it requires no additional reagent and has a high rate constant (0.1−1.0 M^{-1} s^{-1}).[25−28] Because of these advantages,

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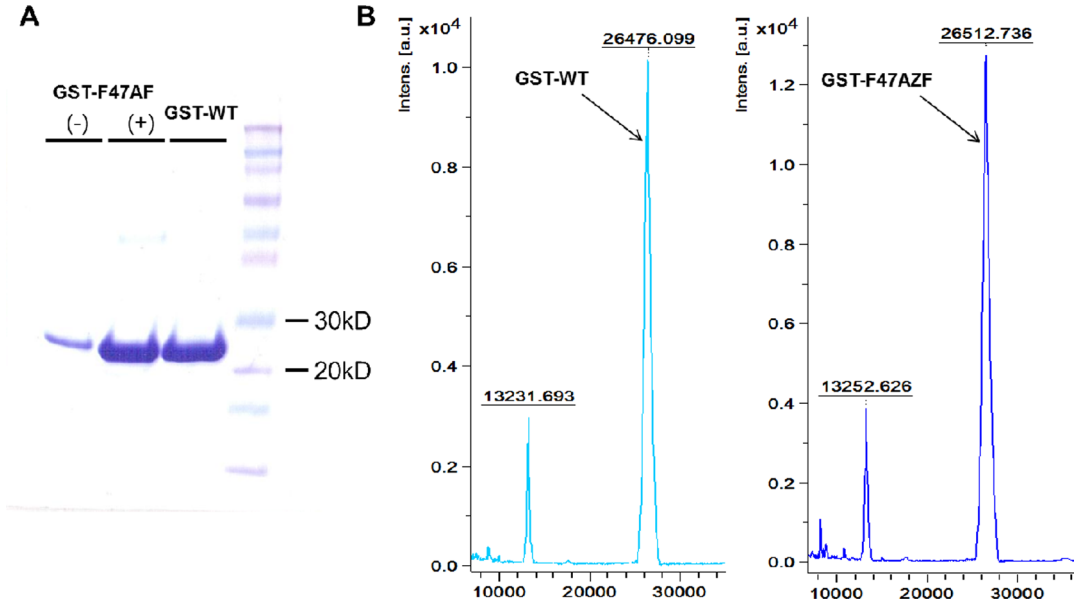


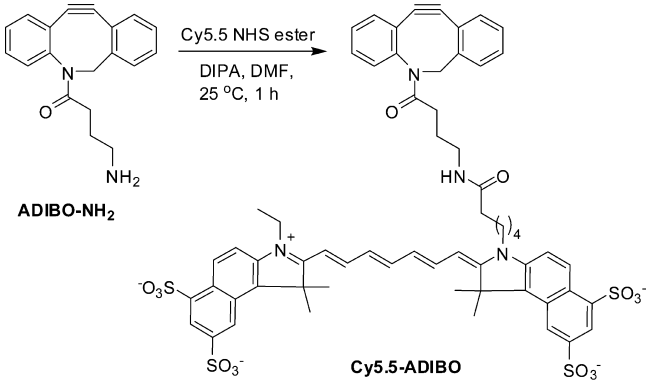
Figure 2. Expression of GST with an amber codon at position 47 (F47) in the presence of the corresponding evolved tRNA/aaRS pair. (A) SDS-PAGE analysis of the mutant GST (GST-F47AZF) and GST-WT; (B) MALDI-TOF mass analysis: expected mass difference between GST-WT (left) and GST-F47AZF (right) = 41 Da; observed mass difference = 37 Da.

SPAAC has been employed for various processes, including labeling proteins and cell surfaces.[29−36] However, in order to apply this method to protein conjugation, the counterpart, azide, must first be introduced into a protein of interest. The incorporation of the azide group into a protein can be achieved by genetic incorporation,[11,12] and it was, therefore, proposed that the application of SPAAC to genetically incorporated azide-containing amino acids would make it possible to label proteins efficiently (Figure 1).

Incorporation of AZF into GST. To introduce the azide group into proteins, *p*-azidophenylalanine (AZF) was chosen as an azide-containing amino acid and glutathione *S*-transferase (GST) as a target protein. Based on the X-ray crystal structure of GST, a solvent-exposed residue, F47, was chosen to be replaced by AZF. To incorporate the amino acid, an amber codon (TAG) was substituted for F47 by site-directed mutagenesis. The GST mutant containing AZF (GST-F47AZF) was expressed in the presence of the corresponding evolved tRNA/amino-acyl tRNA synthetase (aaRS) pair[24] and 1 mM AZF in *E. coli* strain DH10B grown in LB. Mutant and wild-type (WT) proteins were purified by glutathione affinity purification, and the yields of GST-F47AZF and GST-WT were 10−15 mg/L and 15−20 mg/L, respectively. SDS-PAGE analysis showed that a small amount of full-length protein was produced in the absence of AZF (Figure 2A) because of background incorporation. However, MALDI-TOF mass spectrometry (MS) analysis of the purified protein expressed in the presence of AZF confirmed the incorporation of AZF (Figure 2B) with no detectable incorporation of natural amino acids.

GST Conjugation by SPAAC. Next, conjugation of GST-F47AZF with a Cy5.5-linked aza-dibenzocyclooctyne derivative (Cy5.5-ADIBO) was carried out. Cy5.5-ADIBO was prepared from ADIBO-NH_2 (Scheme 1). The amide bond formation reaction with ADIBO-NH_2 and Cy5.5 *N*-hydroxysuccinimide ester (Cy5.5 NHS ester) was carried out in DMF at room temperature for 1 h, and reverse-phase HPLC purification afforded the bioorthogonal azide acceptor in 93% yield. The

Scheme 1. Preparation of Cy5.5-ADIBO as a Bioorthogonal Azide Acceptor



conjugation reaction was performed by adding Cy5.5-ADIBO to the mixture containing the mutant GST in 10 mM phosphate buffer (pH 7.0) and 100 mM NaCl at room temperature. The conjugation reaction was analyzed by SDS-PAGE at different reaction times (Figure 3A,B). Fluorescence images from SDS-PAGE analysis showed that Cy5.5-ADIBO was efficiently conjugated to the mutant GST. The same conjugation reaction with GST-WT showed no fluorescence in SDS-PAGE analysis, meaning that the fluorescence observed in the conjugation reaction with the GST mutant was a result of the specific conjugation of Cy5.5-ADIBO with the azido group incorporated into the target protein (Figure 3A,B). The conjugation reaction was also evaluated by MALDI-TOF MS analysis (Figure 3C). The MS analysis showed that specific conjugation occurred and that approximately 80% of the mutant GST was conjugated. These results show that the AZF-containing protein can be site-specifically conjugated within 120 min by simply mixing the mutant protein with the dibenzocyclooctyne derivative. Compared to the published method (50% labeling yield in 12 h) using cyclooctyne,[19] the reaction time is 6-fold shorter and the labeling yield is 1.6-fold

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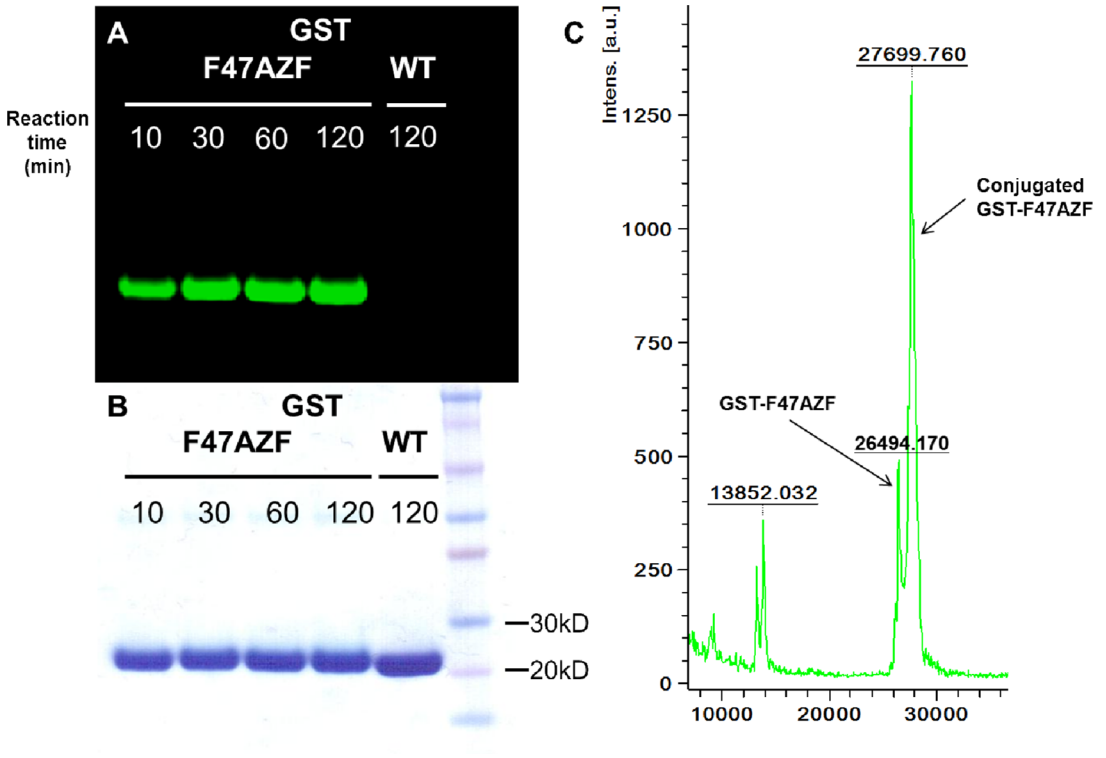

Figure 3. Labeling of GST-F47AZF with Cy5.5-ADIBO. Reaction mixtures were analyzed by SDS-PAGE at different reaction times. Reaction condition: 20 μM GST, 200 μM Cy5.5-ADIBO in 10 mM phosphate buffer (pH 7.0) containing 100 mM NaCl, 10 μL total volume, room temperature. The same reaction was performed with GST-WT and analyzed at 120 min as a control. (A) Fluorescence gel image; (B) Coomassie-stained gel image; (C) MALDI-TOF mass analysis of GST-F47AZF reacted for 120 min: expected mass difference between GST-F47AZF and the conjugated protein = 1190 Da, observed mass difference = 1206 Da.

higher. Although other methods[17,20] can result in slightly better yield, they require harsh reaction conditions or extra steps before labeling as discussed above.

Application to Antibody-Free Western Blot Analysis. The utility of this method was further demonstrated by applying the method to antibody-free Western blot analysis. Western blotting is a powerful analytical technique used to detect a specific protein. While the method provides high specificity and sensitivity, it requires an antibody specific to the protein of interest, and multiple incubation and wash steps for analysis. Therefore, a simple and antibody-free method for protein analysis combining high specificity and sensitivity would be of enormous potential and wide-ranging application. It was proposed that the SPAAC of dibenzocyclooctyne derivatives to genetically encoded AZF could be used for protein analysis, with acceptable specificity and sensitivity.

GST Conjugation with Crude Cell Lysates. To test the specificity of the SPAAC, the same conjugation reaction for purified GST-F47AZF was performed with cell lysate prepared from GST-F47AZF-expressing cells. Cy5.5-ADIBO was added to the cell lysate, and the mixture was incubated for 1 h at 37 °C, followed by SDS-PAGE analysis. The result shows that clear conjugation of the target protein occurs in the lysates from GST-F47AZF-expressing cells, while no conjugation is observed in the lysates from GST-F47AZF-expressing cells grown in the absence of AZF or in those from GST-WT-expressing cells (Figure 4). In addition, minimal background fluorescence was observed from nonspecific conjugation reactions, meaning that the SPAAC is specific to the target protein containing AZF.

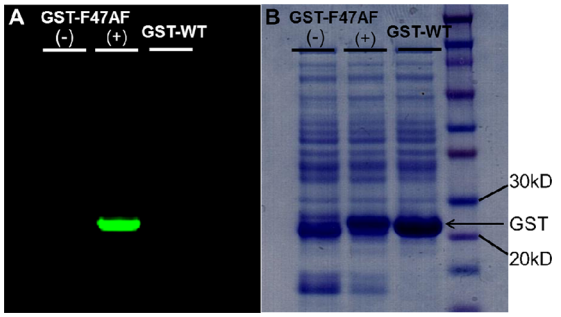

Figure 4. Labeling of GST-F47AZF in crude cell lysate with Cy5.5-ADIBO. Crude cell lysates were prepared from cells expressing GST-WT and GST-F47AZF in the presence (+) and absence (−) of 1 mM AZF. Reaction condition: 10 μL cell lysate, 200 μM Cy5.5-ADIBO in 10 mM phosphate buffer (pH 7.0) containing 100 mM NaCl, 15 μL total volume, 37 °C, 60 min. (A) Fluorescence gel image; (B) Coomassie-stained gel image.

Sensitivity and Utility of the Method. Next, the sensitivity of this method was tested. The product of the reaction between GST-F47AZF and Cy5.5-ADIBO was analyzed by serial dilution. Figure 5 shows that as little as 8–20 ng of labeled protein can be detected. To verify if this method can be used to analyze protein expression levels, GST-F47AZF expression was carried out with different concentrations of L-arabinose. Cell lysates were treated with Cy5.5-ADIBO and analyzed by SDS-PAGE. The results showed clear differences in the expression levels, corresponding to the expression levels expected based on the L-arabinose concentrations provided (Figure 6). Upon comparison, fluorescence

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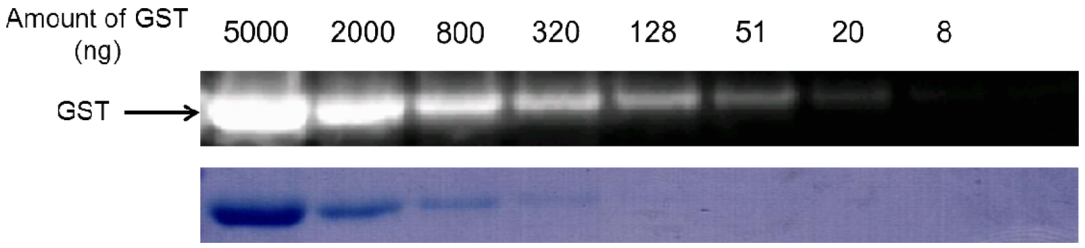

Figure 5. Comparison of gel imaging by fluorescence scanning (top) and Coumassie-staining (bottom) for the GST-Cy5.5-ADIBO conjugate. As little as 8−20 ng of the conjugated protein can be detected by fluorescence scanning.

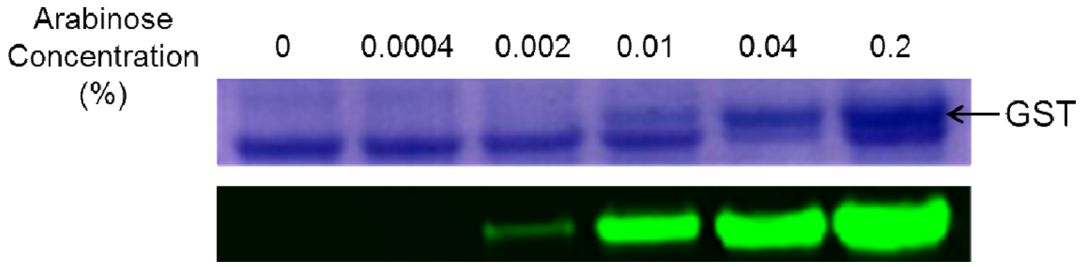

Figure 6. Analysis of protein expression levels by labeling the target protein with Cy5.5-ADIBO. GST-F47AZF is expressed in different concentrations of L-arabinose, the cell lysates were treated with the labeling reagent, and the mixtures were directly analyzed by SDS-PAGE. Top, Coumassie-stained gel image; bottom, fluorescence gel image.

and Coomassie-stained images showed the same patterns of expression. Although this method is not as sensitive as antibody-based Western blotting (which typically detects as little as 0.1 ng when a chemiluminescent substrate is used), it is still much more sensitive than Coomassie staining and appears to have excellent specificity. The most important advantage of this method is that a target protein can be analyzed by simple mixing and SDS-PAGE.

In conclusion, a simple and efficient method for protein labeling was developed in which an azide-containing amino acid was introduced into GST and conjugated with Cy5.5 by SPAAC. This method allows us to label proteins by simply mixing a target protein and a labeling reagent, with a labeling yield of 80% in just 120 min. In addition, the specificity of SPAAC made it possible to analyze the expression levels of a protein quantitatively by simple mixing and SDS-PAGE analysis with no need for antibodies and multistep incubations and washings. Because the genetic incorporation of the azide-containing amino acid can be generally applied to any protein and SPAAC reactions are highly specific, this method should prove very useful for labeling and analyzing proteins.

■ ASSOCIATED CONTENT

Supporting Information

MALDI-TOF-MS spectrum of Cy5.5-ADIBO. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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