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SHORT COMMUNICATION

A modified protein persulfidation detection method

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Abstract

As a gastransmitter, hydrogen sulfide (H_2S) plays an important role in regulating plant growth and stress resistance. The proposed functional mechanism of H_2S is persulfidation, a kind of posttranslational modification on free cysteine residues of proteins, which is also called S-sulfhydration previously. At present, biotin switch assay is an effective method for protein persulfidation level detection, while the protein sample need be precipitated and resuspended repeatedly according to the current protocol. Here, we reported a modified method which adapted from biotin switch assay and reduced the repetitive steps mentioned above. The total protein of plant or purified recombination protein from *E. coli* was directly loaded on the activated NC membrane. The free sulfhydryl of cysteine residues of protein on NC membrane could be blocked with MMTS reagent, while the persulfidation modified cysteine residues would be labeled by biotin-HPDP. After that, the persulfidation level of proteins was detected by immunoblotting with anti-biotin antibodies. This method was also applicable to the plant proteins, which were transferred to NC membrane after separated by native PAGE. This method reduces the repeated steps of precipitation and resuspension of protein samples, and by this it could reduce the loss and content error of protein. Besides, it is more sensitive and convenient than the previous biotin switch assay method.

Keywords Persulfidation detection · Hydrogen sulfide · Biotin switch · Immunoblotting

Abbreviations

DL	Dithiothreitol (DTT)
NC membrane	Nitrocellulose filter membrane
MMTS	S-Methyl methanethiosulfonate
Biotin-HPDP	S-*N*-[6-(Biotinamido)hexyl]-3′-(2′-pyridyldithio) propinamide

Hydrogen sulfide (H_2S) is one of the most popular gas transmitter in the gaseous signal molecule family in recent 20 years. Its important physiologic function in plant development regulating and stress resistance have been verified and confirmed by lots of experiments, through physiological concentration of exogenous H_2S treatment or endogenous H_2S production enzymes coding genes mutants (Kimura 2015; Shi et al. 2015; Pei 2016). Cysteine, the sulfur containing amino acid and essential component for most proteins, is the major substance for H_2S generation in enzyme catalyzing manner. And the cysteine desulphydrase, cysteine desulfurase, β-cyanoalanine synthase and *O*-acetylserine (thiol) lyase have been shown to catalyze the production of endogenous H_2S in plant (Riemenschneider et al. 2005; Birke et al. 2012; Romero et al. 2014).

The cysteine residue is the key functional site for protein maintaining or regulating structure and physiological function, by the means of disulfide bond formation or posttranslational modification on itself (Conte and Carroll 2013). The sulfhydryl (–SH) of cysteine residue in peptides could be oxidized to form –SOH or be nitrosated to form –SNO (S-nitrosylation) (Lu et al. 2013), while the molecular mechanism of H_2S gastransmitter is called S-sulfhydration or persulfidation, a kind of protein posttranslational modification on cysteine residues also, reflected in the change of –SH to –SSH group. This would put up a stronger nucleophilic ability, in other word, greater chemical reactivity for the protein or peptide (Aroca et al. 2017).

The common means for protein persulfidation modification detection are adapted from the detection of protein S-nitrosylation, because of their similarities in the chemical and biological determinants. Both of these two kinds of posttranslational modifications are universal, fundamental

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and abundant, but short lived (Ángeles et al. 2015). The accomplished methods for detecting protein persulfidation level or sites have been summarized (Zhang et al. 2017), including (1) biotin switch assay (Mustafa et al. 2009); (2) cysteinyl labeling assay (Krishnan et al. 2011); (3) maleimide or biotin–thiol assay (Sen et al. 2012; Gao et al. 2015); (4) tag-switch assay (Zhang et al. 2014); (5) mass spectrometry assay (Longen et al. 2016). Here, we report an adapted biotin switch assay method applying to the protein persulfidation level detection.

As shown in Fig. 1a, b, the purified recombinant protein of cucumber (His-Csa5G156220) or arabidopsis, which have been reported to suffer persulfidation by H_2S treatment (Liu et al. 2012, 2019) according to the previous biotin switch assay method (Mustafa et al. 2009), was employed to verify the modified protein persulfidation detection method. Equal amounts of protein samples were incubated with 50, 100 µmol/L NaHS or 1 mmol/L DTT for 30 min at 4 °C, and purified recombinant protein as control. Then, the protein samples were precipitated with same volume acetone at −20 °C for 20 min. After centrifugation under the condition of 4 °C, 2000*g* for 10 min, the proteins were resuspended and quantified in HEN buffer after acetone removal. The NC membrane was soaked in transfer buffer for 10 min and then dried. The resuspended proteins were loaded onto the NC membrane with the amount of 0.5 or 1.0 µg per sample. After placed in darkness for 5 min, the NC membrane was incubated in 20 mmol/L MMTS solution at 50 °C for 30 min to block –SH groups, and then incubated in 2 mmol/L biotin-HPDP solution for 3 h at 25 °C to conjugate biotin on -SSH groups. The NC membrane was washed three times with HENS buffer for 10 min, and then incubated in blocking buffer (5% BSA) for 2 h. The biotin-conjugated protein on NC membrane was detected by immunoblotting with biotin antibody. The results show that the persulfidation levels of the recombinant proteins were increased by NaHS treatment, and DTT-treated protein showed the strongest persulfidation signal without MMTS blocking (Fig. 1a, b).

The persulfidation level of plant protein could also be detected by this protocol. The total proteins of cucumber or arabidopsis were extracted with HEN buffer according to previous report (Jaffrey and Snyder 2001), and then treated with 50, 100 µmol/L NaHS, or 1 mmol/L DTT. After precipitated by acetone and resuspended with HEN buffer, the protein samples were loaded onto the NC membrane and underwent the processes above-mentioned. Obvious persulfidation signals were observed when 0.25, 0.5 or 1 µg protein samples were loaded (Fig. 1c, d). There was scarcely any positive signal on recombinant or plant proteins which were treated by DTT first and then blocked by MMTS.

The total proteins were extracted from cucumber with or without 50 µmol/L H_2S treatment according to Jaffrey's method (Jaffrey and Snyder 2001). 10 mg protein of each sample was loaded and then separated by 12% native polyacrylamide gel electrophoresis. After transferred to NC membrane, the samples were subjected to MMTS solution, biotin-HPDP solution, and immunoblotting assay successively. The result was shown in Fig. 2, the persulfidation level of proteins extracted from NaHS treated cucumber was higher than the proteins from untreated cucumber (Fig. 2).

The results indicated that the process of –SH blocking with MMTS and biotin labelling on –SSH for protein

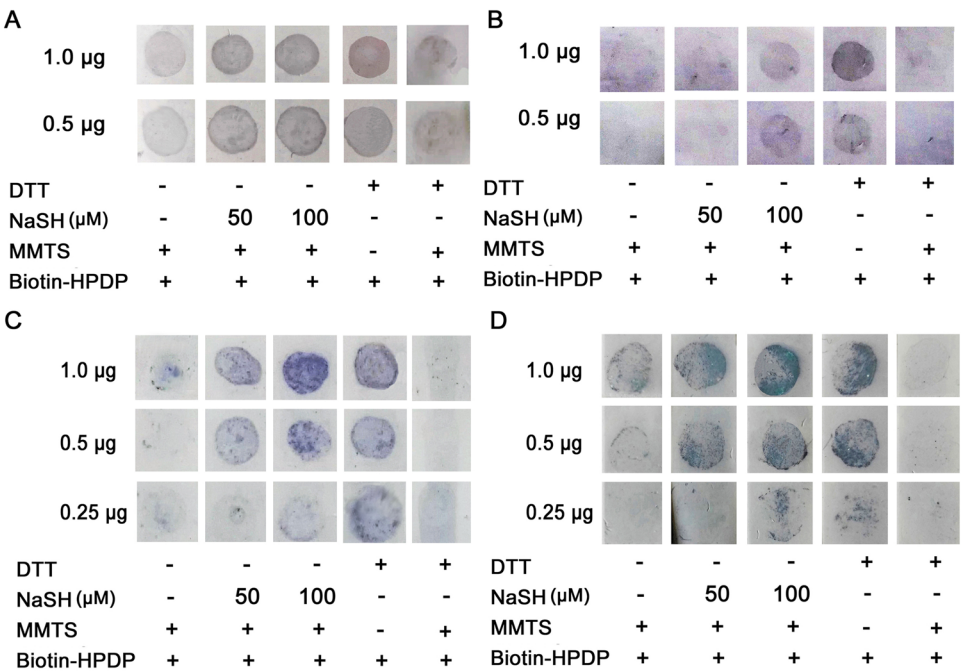

Fig. 1 Persulfidation level detection of recombinant protein or plant total proteins. The 6×His-tagged fusion proteins (His-Csa5G156220) of cucumber (**a**) or arabidopsis (**b**) purified from *E. coli* BL21, or total proteins extracted from leaves of cucumber (**c**) or arabidopsis (**d**) were incubated with 50, 100 µmol/L NaHS or 1 mmol/L DTT for 30 min. After MMTS blocking –SH groups and biotin conjugating to –S–S–H groups, a normal western blot was performed with anti-biotin antibody. DTT (DL-dithiothreitol, 1 mmol/L), MMTS (S-methyl methanethiosulfonate, 20 mmol/L), biotin-HPDP (*N*-[6-(biotinamido) hexyl]-3′-(2′-pyridyldithio) propinamide, 2 mmol/L)

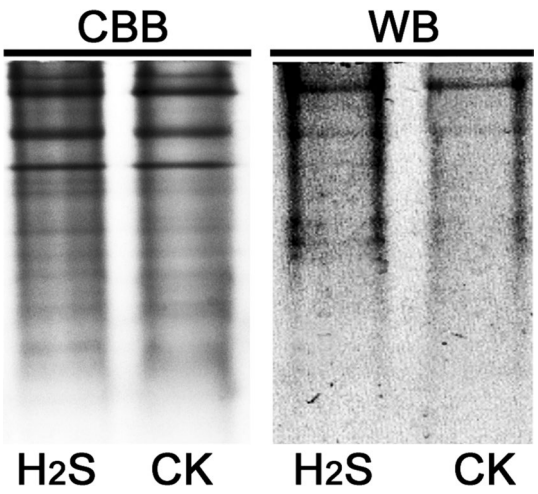

Fig. 2 Cucumber protein persulfidation level detection. Proteins were extracted from leaves of cucumber. *CK* protein from control plant, *H_2S* protein from H_2S fumigated (50 μmol/L, 12 h) plant. 10 mg proteins were separated by native polyacrylamide gel electrophoresis. *CBB* protein separated by native polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue, *WB* western blot of persulfidation modified proteins

persulfidation detection could be performed after protein loading on the NC membrane. According to Mustafa's manuscript, two extra steps of precipitating with acetone and resuspending with HEN buffer need to be performed, after MMTS blocking and biotin-HPDP labelling, while these steps were took out here. Reducing the repeated steps of precipitation and resuspension could reduce the loss and content error of protein samples. Besides, it is more sensitive and convenient than the previous biotin switch assay method.

Reagent

HEN buffer: 250 mM HEPES-NaOH (pH 7.7), 1 mM EDTA, 0.1 mM neocuproine, 100 mM deferoxamine.

MMTS solution: 2 M MMTS dissolved in DMF was diluted to 20 mM with HEN buffer.

Biotin-HPDP solution: 50 mM Biotin-HPDP suspension in DMSO was diluted to 2 mM with HEN buffer.

HENS buffer: HEN buffer with 1% SDS.

Transfer buffer: tris 3 g, glycine 14.4 g, SDS 1 g, dissolved with 800 mL ddH_2O, and then added 200 mL carbinol.

Blocking buffer: 6 mM $Na2HPO_4$, 3.5 mM $KH2PO_4$ and 2.6 mM KCl, pH 7.0 and supplemented with 0.1% Tween-20 (v/v), 1.5% glycine (w/v), 5% BSA (w/v).

Reagent about western

TBST: tris 1.25 g, NaCl 8.76 g, dissolved with 1000 mL ddH_2O, pH 7.5 and supplemented with 0.05% Tween-20 (v/v).

5% BSA (w/v): BSA 5 g dissolved with 100 mL TBST.

Biotin-antibody solution: biotin-antibody was diluted 2000 times with 5% BSA.

AP goat anti-mouse solution: AP goat anti-mouse was diluted 2000 times with TBST.

BCIP: BCIP 500 mg dissolved with 10 mL DMF.

NBT: NBT 500 mg dissolved with 10 mL 70% DMF.

Developer buffer: tris 12.114 g, NaCl 5.844 g, $MgCl_2{\cdot}6H_2O$ 1.0116 g, dissolved with 1000 mL ddH_2O, pH 7.5.

Developer solution: 33 μL BCIP, 66 μL NBT, dissolved with 10 mL developer buffer.

Author contribution statement The idea of experiment came from ZQL and the process was guided by ZQL. YQJ and YWL carried out this experiment. CYC prepared reagents for this work. Some assistance was gave by JJQ in the process of purifying protein. YXP reviewed and examined the experiment.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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