A simple semi-quantitative in vivo method using H_2S detection to monitor sulfide metabolizing enzymes

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BioTechniques 57:208-210 (October 2014) doi 10.2144/000114218
Keywords: lead acetate; sulfide:quinone oxidoreductase; decylubiquinone; microtiter plate

Supplementary material for this article is available at www.BioTechniques.com/article/114218.

Here we present a simple in vivo microtiter plate assay using lead acetate [$Pb(OAc)_2$]–soaked filter paper to detect H_2S released by *Escherichia coli* metabolizing cysteine. The released H_2S precipitates as brown lead sulfide (PbS) on $Pb(OAc)_2$ soaked filter paper. The PbS stain quantitated by ImageJ software is proportional to the amount of H_2S released from the culture. Expression of recombinant *Acidithiobacillus ferrooxidans* sulfide:quinone oxidoreductase (SQR) converts the H_2S to sulfur, resulting in less PbS formation. The in vivo H_2S oxidation activity of SQR was calculated based on the density of the PbS stain formed by *E. coli* expressing SQR compared with cells harboring the empty vector pLM1. The results are consistent with the in vitro activity of SQR measured by decylubiquinone (DUQ) reduction. This assay can be applied to sulfide metabolizing enzymatic studies, mutant screening and high-throughput inhibitor screens.

We use lead acetate [$Pb(OAc)_2$]–soaked filter paper to measure the in vivo activity of heterogeneously expressed sulfide:quinone oxidoreductase (SQR) based on the reaction of volatile H_2S with $Pb(OAc)_2$ to form insoluble lead sulfide (PbS). The PbS stain is proportional to the amount of H_2S released from the culture. The in vivo H_2S oxidation activity was calculated based on the color density of the brown PbS stain formed by *E. coli* expressing SQR compared with cells harboring the empty vector pLM1.

Sulfide (existing as three different forms: H_2S, HS^-, and S^{2-}) is involved in a variety of biological processes. (In this paper the term "sulfide" will refer to the total sulfide present in solution including the three different forms: H_2S, HS^-, and S^{2-}. The species H_2S, SH^-, and S^{2-} will be named specifically as necessary.) In mammals, sulfide is a very toxic molecule that inhibits mitochondrial ATP production (1). However, it can be used as an energy source by microbes (2). In recent years, sulfide has been identified as a signaling molecule that plays prominent roles in cellular physiology and pathophysiology, including the regulation of vascular homeostasis, inflammation, apoptosis, and cellular stress response (3–5).

Several enzymes are involved in sulfide metabolism. Sulfide is produced from cysteine and/or homocysteine by sulfide-generating enzymes, for example, cystathionine β-synthase (EC 4.2.1.22), cystathionine γ-lyase (EC 4.4.1.1), 3-mercaptopyruvate sulfurtransferase (EC 2.8.1.2) teamed with cysteine aminotransferase (EC 2.6.1.3), cysteine lyase (EC 4.4.1.10), and cysteine desulfurase (EC 2.8.1.7) (6–9). Sulfide is membrane permeable and highly diffusible in its gas form (H_2S) (10). Enzyme-produced sulfide can be removed as sulfane-bound sulfur, which is the storage intermediate for sulfide and in turn may release sulfide when cells are under certain physiological conditions (11). SQR (EC 1.8.5.4), acting as a sulfide remover, catalyzes the oxidation of sulfide to elemental sulfur (polysulfide or octasulfur rings) (12). Thiol S-methyltransferase (EC 2.1.1.9) and thiosulfate:cyanide sulfurtransferase (EC 2.8.1.1) are also involved in removing sulfide via methylation and oxidation, respectively (13,14).

$Pb(OAc)_2$, mercuric chloride or silver nitrate impregnated paper strips are widely used to qualitatively detect H_2S in the atmosphere and in solution with high sensitivity (15). These strips are also used for the qualitative detection of microbial H_2S production by inserting a $Pb(OAc)_2$–soaked paper strip between the plug and inner wall of a culture tube, above the inoculated medium (16). The major conventional methods for sulfide quantitative measurement in biological samples are colorimetric (e.g., the methylene blue method), electrochemical (sulfide-selective electrodes), gas chromatography, and sulfide precipitation–analysis (17). However, these methods require sample homogenization and/or acidification prior to analysis. Fluorescent probe-based

METHOD SUMMARY

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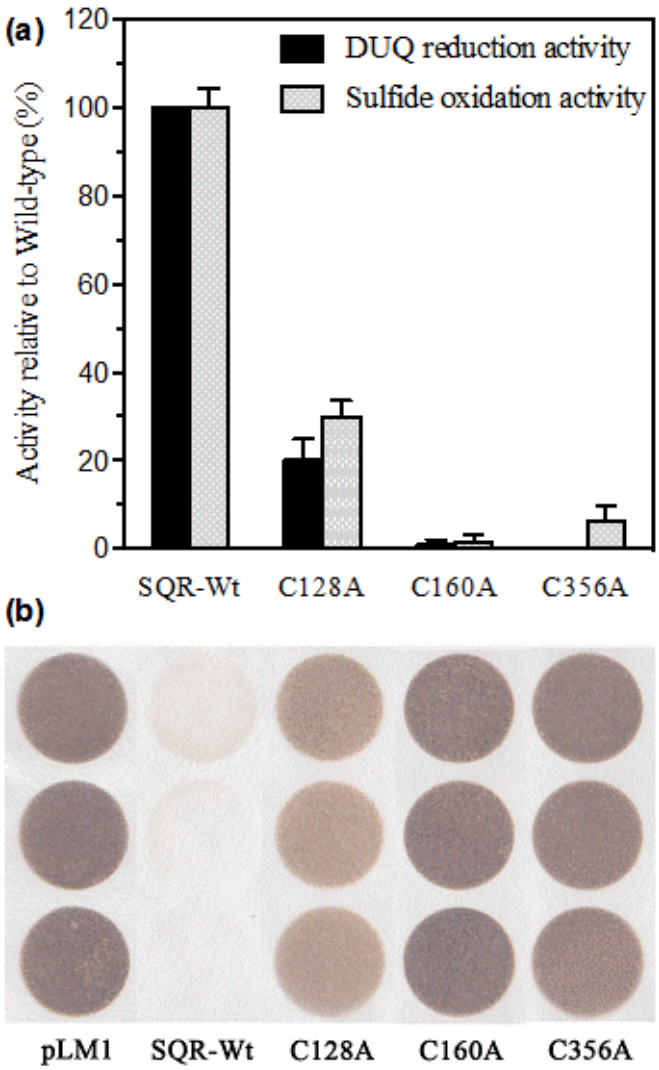

Figure 1. Comparison of decylubiquinone (DUQ) reduction activity with lead sulfide (PbS) precipitation. (a) Relative enzyme activities and PbS stain formed by wild-type (Wt) and variant sulfide:quinone oxidoreductase (SQR). One hundred percent DUQ reduction activity is 8.07 µmol mg^{-1} min^{-1} (21). One hundred percent H_2S oxidation activity is the differential density (differential mean gray value = 56.91 ± 3.74) of the PbS stain formed by *E. coli* BL21(DE3) harboring wild-type SQR and the empty vector pLM1. The mean gray values were measured using consistent area selection and normalized to the A_{600} of the culture. The relative activity compared with the wild-type SQR was calculated using the A_{600} normalized mean gray values. Relative activity (%) = (the mean gray value of stain X - the mean gray value of stain pLM1) × 100 / (the mean gray value of stain wild-type - the mean gray value of stain pLM1). The mean values of three experiments are shown; error bars indicate standard deviation. (b) PbS stain formed by *E. coli* BL21(DE3) harboring the empty vector pLM1, SQR-Wt, and SQR variants. As the in vivo H_2S oxidation activity assay is not measured as a function of time, an activity 10% or less that of wild-type was considered as a total loss of activity.

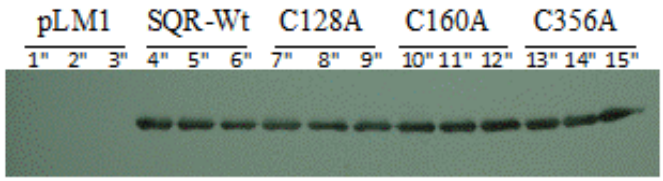

Figure 2. Western blot analysis of His-tagged sulfide:quinone oxidoreductase (SQR) expression levels in *E. coli* BL21(DE3) transformed with empty vector, a vector expressing wild-type SQR, or SQR variants. Culture samples (0.2 mL) were taken from each well and pelleted by centrifugation at 10,000 rpm. The pellets were suspended in 80 µL 1× protein sample loading buffer supplemented with fresh dithiothreitol (DTT). The suspended samples were boiled for 10 min and centrifuged at 10,000 rpm for 2 min. Ten µL of supernatant from each sample was analyzed by Western blot immunodetection of the His-tag using monoclonal mouse antibody (Qiagen) and anti-mouse secondary IgG (Qiagen).

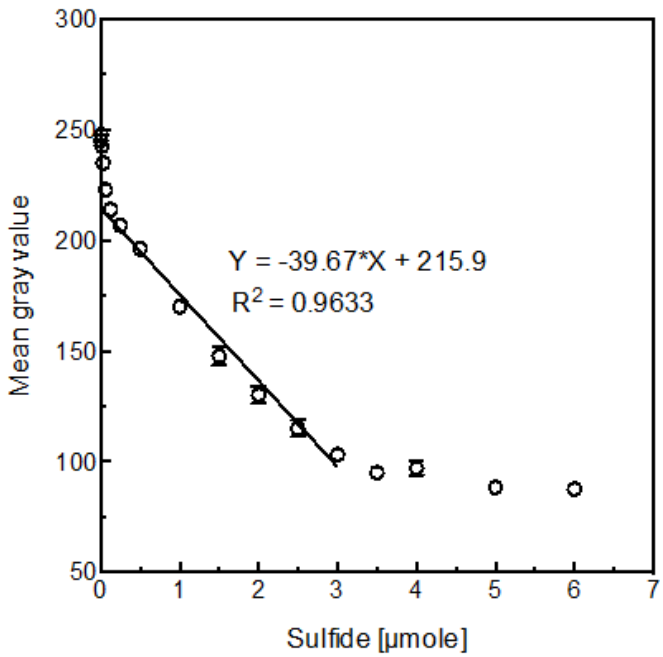

Figure 3. Calibration curve for sulfide. Na_2S solutions (20 µL), with concentrations varying from 0 to 300 mM, were dripped on pre-dried 2% $Pb(OAc)_2$ soaked filter paper. The lead sulfide (PbS) stains formed were scanned and quantified by measuring the mean gray value with ImageJ software (20) using consistent area selection. The mean gray values were plotted against the amount of sulfide (in moles) dripped onto the $Pb(OAc)_2$ soaked filter paper. A linear relationship was obtained over the range of 0.0625–3 micromoles. The correlation coefficient (R^2) was 0.9633.

methods can be very suitable tools for detecting, measuring, and visualizing sulfide species in biological systems due to their high sensitivity, selectivity, and real-time capability (18). The in vivo activities of enzymes are normally examined by monitoring their abilities to support growth in minimal medium supplemented with the corresponding substrates (19). However, this method is not suitable for sulfide metabolizing enzymes as sulfide is a regulator and not essential for cell growth.

Here we present a simple in vivo activity assay method for recombinantly-expressed sulfide metabolizing enzymes in *E. coli*. Metabolism of cysteine in *E. coli* produces sulfide, which is highly diffusible in its gas form (H_2S) (10). The released H_2S precipitates as PbS on $Pb(OAc)_2$–soaked filter paper. Cells expressing functional SQR can remove or store the sulfide by converting it to inorganic sulfur (9,12), resulting in less PbS precipitate. A detailed protocol is available in the Supplementary Material. Briefly, a 2% overnight culture of *E.*

coli BL21(DE3) transformed with empty vector, a vector expressing His-tagged wild-type SQR, or SQR variants with reduced activity, was inoculated into Luria Bertani (LB) medium, containing 100 mg/L ampicillin and 300 µM isopropyl-β-D-thiogalactopyranoside (IPTG), and grown at 37°C for 3 h with shaking at 180 rpm. Freshly prepared cysteine (250 µM) was added to the culture, and 1 mL was transferred to a well in a 24-well tissue culture microtiter plate. The lid was covered with a piece of filter paper soaked in 2% $Pb(OAc)_2$. The plate was placed in a 30°C shaker for 17 h with shaking at 180 rpm. H_2S gas released from cysteine metabolism reacts with the $Pb(OAc)_2$ in the moistened filter paper to form a brown PbS stain. The PbS stain is proportional to the amount of H_2S released from the culture. The stained paper was scanned and quantified by measuring the mean gray value using ImageJ software (20). The results were normalized to the A_{600} of the culture. H_2S oxidation activity was calculated based on the color density of the PbS stain formed by *E. coli* expressing SQR compared with cells harboring the empty vector pLM1. The relative activity compared with the wild-type was calculated using the A_{600} normalized mean gray values. The relative activity (%) = (the mean gray value of stain X - the mean gray value of stain pLM1) × 100 / (the mean gray value of stain wild-type - the mean gray value of stain pLM1).

Figure 1 summarizes the results, which reflect the in vitro activity of SQR measured by decylubiquinone (DUQ) reduction assays (21). *E. coli* BL21(DE3) expressing wild-type SQR completely oxidized endogenously-produced H_2S during growth, thus preventing the precipitation of PbS on the $Pb(OAc)_2$–soaked paper. In contrast, H_2S released from *E. coli* BL21(DE3) harboring the empty vector pLM1 reacted with $Pb(OAc)_2$ soaked filter paper and formed insoluble PbS stain. A variant, $SQR^{Cys128Ala}$, which retained 30% DUQ reduction activity, displayed similar activity based on PbS precipitation. Inactive $SQR^{Cys160Ala}$ and $SQR^{Cys36Ala}$ variants were inactive in both assays. The differences in the in vivo sulfide oxidation activity of the overexpressed SQR mutants were not due to different expression levels, as indicated by Western blotting (Figure 2).

In conclusion, the method described here provides a simple and efficient way to measure the in vivo activity of recombinantly-expressed sulfide metabolizing enzymes in *E. coli*. Our approach is simple and can be used for sulfide metabolizing enzyme studies, mutant screening, and high-throughput inhibitor screens. The limit range for released sulfide is 0.0625–3 micromoles (Figure 3). The PbS stain reaches saturation when more than 3 micromoles of H_2S is released from each well of a 24-well tissue culture microtiter plate.

Author contributions

Y.Z. carried out the experiments, performed the data analysis, and drafted the manuscript. J.H.W. conceived of the study, supervised the research, and helped to draft the manuscript. All authors read and approved the final manuscript.

Acknowledgments

This work was funded by the Canadian Institutes of Health Research (CIHR MOP89735).

Competing Interests

The authors declare no competing interests.

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Received 18 June 2014; accepted 10 September 2014.

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