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Enhanced Detection of β -Galactosidase Reporter Activation Is Achieved by a Reduction of Hemoglobin Content in Tissue Lysates

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ABSTRACT

β -galactosidase (β -gal), the product of the *E. coli* LacZ gene, has been used extensively as a reporter in numerous systems. Until recently, the most commonly used method of detecting β -gal reporter enzymatic activity was a colorimetric assay based on the cleavage of the β -gal substrate 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside (X-gal) to form a blue precipitate. However, when increased sensitivity is needed, many investigators now turn to alternate substrates that produce fluorescent or luminescent products upon cleavage by β -gal. These products are much more easily quantified than X-gal. The luminescent and fluorometric assays work very well in cultured cells but are often less sensitive in whole tissue lysates. In this study, we have evaluated the sensitivity of a fluorescent and a luminescent substrate in whole tissue lysates cleared of red blood cells or washed with PBS only. We have found that both assays show increased low-end sensitivity in tissues with reduced levels of hemoglobin (Hb). Hb is apparently able to quench luminescent and, to a lesser degree, fluorescent reporter light emission. Therefore, steps should be taken to reduce Hb levels either by lysis, perfusion, or both to enhance the sensitivity of these assays.

INTRODUCTION

The β -galactosidase (β -gal) gene has been widely used as a reporter enzyme in the evaluation of gene regulation, detection of recombinant fusion genes, monitoring of cell transfection efficiencies, and identification of protein-protein interactions by the two-hybrid system (5,8). Although mammalian cells contain endogenous β -gals, these enzymes are generally lysosomal with

low-pH optima and low activity at neutral pH (7,10). Furthermore, significant reductions of endogenous but not bacterial activity can be achieved using heat inactivation (10). These characteristics make bacterial β -gal an attractive reporter system. One of the most popular substrates used to monitor β -gal activity resulting from *LacZ* gene expression has historically been 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside (X-gal). This substrate is cleaved by β -gal to form a blue precipitate and can be used to localize reporter signal within a tissue. However, this system is limited in sensitivity, and the reporter signal is difficult to quantify.

In recent years, a number of alternative substrates for β -gal that produce luminescent or fluorescent products upon cleavage by β -gal have been introduced. Examples of assay kits based on such substrates include Galacto-Light Plus™ (Applied Biosystems, Foster City, CA, USA) and FluoReporter® (Molecular Probes, Eugene, OR, USA), respectively (1,2,6). Both of these assay systems provide very sensitive detection of β -gal activity in cultured cells. However, we have found that sensitivity tends to be lower and background higher in whole tissue extracts. This may mask small changes in reporter enzyme activation, underestimate enzymatic activity, and increase the amount of variability. It is possible that high concentrations of lipid or protein in tissue extracts may negatively influence sensitivity. Hemoglobin (Hb) in particular has been previously found to interfere with the chemiluminescent reporter activity of alkaline phosphatase (1,3). We have noticed that lysates that appear red were routinely lower in reported β -gal activity. We also found that the addition of a solution containing red blood cell (RBC) lysate reduced the amount of β -gal activity detected in tissue lysates. We evaluated the role of Hb interference in the luminescent and fluorescent systems for β -gal using extracts from tissues obtained from transgenic animals expressing β -gal (9) or in a system containing purified β -gal and Hb only. The tissues had either been reduced in RBCs by lysis with an ammonium chloride, potassium bicarbonate, and EDTA solution (AcK) or by thorough washing with PBS only. We have

determined that the presence of Hb can interfere with the sensitive detection of β -gal enzymatic activity in tissue extracts and that simple steps to reduce the Hb content in these samples can significantly improve both sensitivity and background. We are not aware of any β -gal activity assessment protocols that specify the need to remove Hb. Data presented herein suggest that an important addition to these protocols would include steps to reduce Hb content through the lysis of RBCs with AcK followed by washing and/or the perfusion of tissues before lysates are obtained. Without such steps, these assays may routinely underestimate enzymatic activity.

MATERIALS AND METHODS

Preparation of Purified β -Gal/Hb Solutions

Purified β -gal and Hb (Sigma, St. Louis, MO, USA) were used to confirm the role of Hb in a simplified system. Varying amounts of Hb and β -gal were mixed in a lysis solution containing 100 mM potassium phosphate, 0.2% Triton® X-100, 1 mM dithiothreitol (DTT), 0.2 mM PMSF, and 5 μ g/mL leupeptin (Solution A). The DTT, leupeptin, and PMSF were added to the solution just before use. These samples were then heated at 48°C for 60 min, which is equivalent to heating of tissue extracts to inactivate endogenous β -gal activity, and stored at -70°C. The luminescence of 5- μ L lysate samples was assayed with the Galacto-Light Plus kit and read for 5 s on a Turner model 20e luminometer (Turner Designs, Sunnyvale, CA, USA). Fluorescence of 10- μ L lysate samples was evaluated using the FluoReporter system. The samples were quantified using a Fluoro-Count™ microplate reader (Packard Instruments, Meriden, CT, USA).

Tissue Extract Preparation, Clearance of Hb, and Assay of β -Gal Activity

A biochemical assay based on the enzymatic cleavage of a β -gal substrate to produce a luminescent or fluorescent product was used to assay β -gal activity

in tissues as well. Tissues were harvested from mice expressing the inducible *LacZ* transgene and washed in PBS (9). RBCs were then lysed by incubation for 5 min in a hypo-osmotic AcK solution, or the tissues were washed with PBS only. Once in the AcK solution, the tissues were cut into small pieces (about 5 mm) to allow enhanced access to the lysis buffer. The tissue was then washed with PBS and resuspended in Solution A. The tissue was allowed to incubate in this buffer for 5 min on ice and was then homogenized. The suspension was transferred to a microcentrifuge tube and centrifuged for 2 min at 12000× g. The supernatant was heated at 48°C for 60 min to inactivate mammalian (endogenous) β -gal activity (10). The lysates were then stored at -70°C. The luminescence and fluorescence were determined as described in the previous section.

Protein Determination

Protein concentration was determined for tissue extracts using the NanoOrange® protein quantitation kit from Molecular Probes, according to the manufacturer's instructions.

Quantitation of Hb

The relative concentration of Hb in tissue extracts was determined using the cyanomethemoglobin method of Drabkin and Austin (4). The procedure is based on the oxidation of Hb to methemoglobin in the presence of alkaline potassium ferricyanide to form cyanomethemoglobin. The absorbance of the cyanoderivative is then determined at 540 nm. The results were compared to a standard curve generated using methemoglobin reconstituted in Drabkin's reagent. Drabkin's reagent and the methemoglobin standard were obtained from Sigma.

RESULTS

The initial assessment of the impact of Hb on purified β -gal enzymatic activity was performed utilizing purified β -gal and Hb in Solution A. Samples containing 1.4 g/dL Hb showed a lower level of luminescent reporter gene activation over a range of β -gal concentrations as compared to samples that contained no Hb (Figure 1A). Differences between samples with and without Hb

were significant ($P \leq 0.005$) even at the lowest concentration of β -gal, indicating that sensitivity is reduced by the presence of Hb. A significant difference is detected between 0 and 0.2 U β -gal in the no Hb sample ($P = 0.001$). This difference does not become significant for the Hb containing samples until 2 U β -gal in both the luminescent and fluorescent assays ($P < 0.001$). However, the presence of Hb had only a slight effect on the fluorescent assay system (Figure 1B).

To determine if there is a dose response effect of Hb on the β -gal fluorescent and luminescent endpoints, the effect of increasing concentrations of Hb in the presence of 10 U β -gal was evaluated. Hb was able to significantly reduce detected luminescent β -gal activity at doses as low as 0.1 g/dL, with this effect apparently reaching a maximum near 0.4 g/dL (Figure 2A). Detection of the fluorescent product resulting from β -gal enzymatic activity was also reduced by increasing concentrations of Hb (Figure 2B).

The negative effects of Hb on these assay systems are best addressed by taking steps to remove Hb from the system. In Figure 3, the results of processing tissue from the same induced β -gal transgenic animal with and without AcK lysis of RBCs are presented. These results show relative light units (RLU) of 41.0 (± 0.14) with AcK lysis and of 30.6 (± 0.2) without reduction of Hb by RBC lysis. Background activities were 5.2 (± 0.1) and 5.1 (± 0.1) for the lysed and non-lysed samples, respectively.

DISCUSSION

The sensitivity of luminescent substrates such as Galacton-Plus® (Applied Biosystems) and fluorometric substrates such as 3-carboxyumbelliferyl β -D-galactopyranoside (CUG) (Molecular Probes) are much greater than that of X-gal when used to evaluate cell or tissue lysates. The primary limitation of these systems is the fact that specific cellular localization of reporter activation within a tissue cannot be determined. Additionally, the sensitivity of these assays is limited in whole tissue extracts. Previously, it has been shown that lipid concentration, protein

Figure 1. The presence of purified hemoglobin inhibits β -gal enzymatic activity as measured by both luminescent and fluorescent endpoints. (A) Samples containing 1.4 g/dL Hb and increasing concentrations of β -gal show lower detected enzymatic activity than control samples without Hb as determined using the Galacton-Plus substrate. The differences between Hb/no Hb samples were significant at the lowest β -gal concentration evaluated. ANOVA statistics ** = $P < 0.005$; * = $P < 0.001$. (B) The same samples showed little difference with or without Hb when a fluorometric (CUG) assay was utilized. Only the 10-U sample showed any degree of difference (ANOVA *** = $P < 0.01$). (#) A significant difference is detected between 0 and 0.2 U β -gal in the no Hb sample ($P = 0.001$). This difference does not become significant for the Hb-containing samples until 2 U β -gal in both the luminescent and fluorescent assays ($P < 0.001$).

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concentration, and particularly Hb levels can possibly interfere with luminescent-based assays of reporter enzyme activity (3). Despite this information, few protocols include any steps to minimize the impact of Hb on such chemiluminescent assays. In this study, we present evidence that Hb does reduce the chemiluminescent reporter signal of luminescent β -gal substrates and to a lesser degree the fluorescent emission from β -gal-cleaved substrates.

To support our suspicion that a component of blood was influencing detected enzymatic activity, we added a blood preparation that contained Hb to liver lysates to determine if this would influence the detected enzymatic activity. This experiment indicated that reporter enzymatic activity decreased with increasing proportions of blood. It was likely that the Hb in the blood was causing this effect. However, it could also be the protein concentration or lipid in the lysates. This issue was addressed in a simplified system containing only Hb and β -gal in Solution A. Hb was again shown to reduce luminescent signal. This Hb effect was observed at the lowest concentrations evaluated and appears to be dose dependent up to about 0.8 g/dL Hb. The 0.1 g/dL Hb dose, which is routinely achieved by AcK lysis, showed the lowest reduction in luminescent activity. A significant difference from zero is detected at 0.2 U β -gal in the no Hb sample and at 2 U β -gal in the samples containing Hb (Figure 1). It is possible that the presence of Hb contributes to a basal level of luminescence, which could decrease low-end sensitivity. Reduction of Hb content apparently lowers this background signal, improving signal-to-noise ratios.

ROSA26 animals constitutively express high levels of β -gal in all tissues (11). Addition of purified Hb to ROSA26 liver lysates decreases the observed β -gal signal. Additionally, tissues from ROSA26 animals showed higher levels of β -gal induction if they were first cleared of RBCs by AcK lysis or perfusion (data not shown), indicating that even very high-expressing systems such as the ROSA26 animals can be affected by the presence of Hb. Perfusion of the liver did produce similar luminescence readings compared to AcK alone,

Figure 2. Inhibition of purified β -gal enzymatic activity is similar with increasing concentrations of hemoglobin. (A) A reduction in β -gal enzymatic activity is detected at the dose of 0.1 g/dL Hb. This effect reaches a maximum at 0.4 g/dL hemoglobin. ANOVA statistics ** = $P < 0.005$; * = $P < 0.001$. There is also a significant difference between the 0.1 g/dL Hb and the 0.4 g/dL Hb samples by ANOVA ($P = 0.001$). The 0.1 g/dL Hb level is routinely achievable by AcK lysis. (B) The same samples showed a less significant difference when a fluorescent endpoint for β -gal was evaluated. ANOVA *** = $P < 0.01$.

Figure 3. Reduction of Hb by RBC lysis results in higher RLU. Liver from transgenic animals expressing a dioxin responsive *LacZ* reporter construct was evaluated for β -gal induction using the Galacto-Light Plus luminescent assay. Dioxin-treated liver lysates were compared to lysates from untreated animals. Lysis of RBCs using AcK followed by a PBS wash resulted in a better fold induction of the *LacZ* reporter (7.8 for AcK and 6.0 for no AcK) and higher RLU values than PBS alone. There was no significant difference in control values. The concentration of Hb was lowered to a level of 0.1 g/dL (0.5 g/dL for the non-lysed). * = significant difference by ANOVA ($P = 0.001$).

indicating that it is likely the reduction in Hb content that causes the increased sensitivity of the assay and not an activity of AcK itself. However, not all tissues are as readily perfused as liver. Therefore, a general method such as RBC lysis that helps deplete Hb content has practical advantages.

We have shown that very simple steps to reduce the amount of Hb in tissue extracts can significantly improve upon both the sensitivity and signal-to-noise ratio of these assays. The use of AcK to lyse RBCs lowers the concentration of Hb in samples to levels that are less inhibitory of chemiluminescent signal (Figure 2A and Figure 3), and perfusion of the tissues also enhances chemiluminescence (not shown). It is therefore important to consider the potential influence of Hb on both luminescent and fluorescent assays for β -gal activity and to take steps to minimize the influence of Hb by RBC lysis and/or perfusion of tissues. Additionally, Hb is likely to have a similar effect on other reporter gene assays that are dependent on chemiluminescence.

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