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A Simple Assay for Mammalian Spermine Oxidase: A Polyamine Catabolic Enzyme Implicated in Drug Response and Disease

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Abstract

Spermine oxidase (SMO), the most recently characterized polyamine metabolic enzyme, catalyzes the direct back-conversion of spermine to spermidine in an FAD-dependent reaction that also yields the byproducts hydrogen peroxide (H_2O_2) and 3-aminopropanal. These metabolites, particularly H_2O_2 , have been implicated in cytotoxic cellular responses to specific antitumor polyamine analogs, as well as in the inflammation-associated generation of DNA damage. This chapter describes a rapid, sensitive, and inexpensive method for the chemiluminescent measurement of SMO (or alternatively, N^1 -acetyl polyamine oxidase, APAO) enzyme activity in cultured cell lysates, without the need for radioactive reagents or the use of high performance liquid chromatography (HPLC). Specifically, H_2O_2 production by SMO is coupled to chemiluminescence generated by the horseradish peroxidase-catalyzed oxidation of luminol. Detailed protocols for preparation of reagents, harvesting cell lysates, generation of a standard curve, assaying of samples, and calculation of SMO enzyme activity are presented.

Keywords

Spermine oxidase; Polyamine catabolism; Hydrogen peroxide; Chemiluminescence

1. Introduction

While the enzymatic biosynthesis of polyamines is irreversible, a group of tightly regulated, complementary enzymes facilitate the back-conversion of the higher polyamines spermine and spermidine to spermidine and putrescine, respectively (1). The first pathway involves acetylation of spermine or spermidine to N^1 -acetylspermine or N^1 -acetylspermidine by spermidine/spermine N^1 -acetyltransferase (SSAT) (2). The acetylated polyamine then undergoes oxidation to spermidine or putrescine by acetyl polyamine oxidase (APAO), with generation of hydrogen peroxide (H_2O_2) as a byproduct (3, 4). Alternatively, spermine oxidase (SMO), the most recently characterized polyamine catabolic enzyme (5, 6), catalyzes the direct back-conversion of spermine to spermidine, 3-aminopropanal, and H_2O_2 , without an acetylated intermediate. This stoichiometric production of H_2O_2 by SMO facilitates quantification of SMO enzyme activity by the chemiluminescent method described in this chapter.

The production of H_2O_2 by SMO and APAO activity has been associated with downstream oxidative damage and has important implications in the etiology and treatment of human diseases (7–9). The cytotoxic effect of numerous polyamine analogs under investigation as chemotherapeutic agents has been ascribed to the generation of reactive oxygen species, resulting from massive upregulation of polyamine catabolism through the SSAT/APAO pathway (10–12). Recent studies, however, suggest that SMO is the more critical source of

H₂O₂ with respect to analog-induced cell death (13, 14). Furthermore, oxidative damage following modest upregulation of SMO in epithelial tissues by inflammatory stimuli is hypothesized to contribute to inflammation-associated carcinogenesis (reviewed by (1)). The following protocol, based on a method originally reported by Fernandez et al. (15), has been altered to provide greater sensitivity, allows for real-time analysis of polyamine oxidase activity (SMO or APAO) (16, 17), and provides the potential for modification to high-throughput applications, such as the screening of potential antitumorigenic compounds for induction of oxidase activity in cancer cell lysates.

In preparation for the measurement of SMO activity, cells are harvested in glycine buffer and lysed by freezing at -80°C . The assay reaction mix contains cell lysate, horseradish peroxidase (HRP), luminol, pargyline (monoamine oxidase inhibitor), and aminoguanidine (diamine oxidase inhibitor), in a glycine buffer at pH 8.0. This reaction is incubated briefly at 37°C , followed by addition of spermine (substrate for SMO) and integration of chemiluminescence. The slightly basic pH (8.0) permits the HRP to catalyze the oxidation of luminol in a reaction coupled to the production of H₂O₂ by SMO. Upon oxidation of luminol, a light-emitting molecule (excited 3-aminophthalate ion) is produced (Fig. 1). The amount of chemiluminescence measured in a sample corresponds to the relative oxidation of spermine by SMO in the cell lysate.

2. Materials

2.1. Reagents That May Be Prepared in Advance and Stored at -20°C

1. Aminoguanidine (Sigma #A7009; FW: 123.1 g/mol). Dissolve 123.1 mg/10 mL HPLC-grade water to yield 100 mM stock solution; aliquot in 1.5 mL tubes and store at -20°C .
2. Luminol (3-aminophthalhydrazide; Fluka Biochemika #09253; FW: 177.16 g/mol). Dissolve 177.2 mg/10 mL dimethyl sulfoxide (DMSO) to yield 100 mM stock solution; aliquot in light-protected 1.5 mL tubes and store at -20°C .
3. Pargyline (*N*-methyl-*N*-(2-propynyl)benzylamine hydrochloride; Sigma #P8013). Dissolve in HPLC-grade water to yield 2.3 mg/mL stock solution; aliquot in 1.5 mL tubes and store at -20°C .
4. Spermine tetrachloride (see Note 1) (Sigma #S2846; FW: 348.19 g/mol). Dissolve 52.2 mg/10 mL HPLC-grade water to yield 15 mM stock solution; aliquot in 1.5 mL tubes and store at -20°C . On the day of the assay, dilute to a 1.5 mM working stock with HPLC-grade water.

2.2. Reagents That Must Be Prepared Fresh for Each Assay

1. Glycine buffer (J.T. Baker# 4059-02; FW: 75.07 g/mol). Dissolve 375.4 mg/10 mL HPLC-grade water to yield 0.5 M stock solution and adjust pH to 8.0 with NaOH. Make a further dilution to 0.083 M (1 part 0.5 M glycine, pH 8.0 plus 5 parts HPLC-grade water) for harvesting cells and enzyme assay blank (see Note 2).
2. HRP (Roche #10814407001, EIA grade, lyophilized). Dissolve in HPLC-grade water to yield a 0.4 mg/mL solution (see Note 3).
3. H₂O₂ (3% w/v solution) (see Note 4).

¹Alternatively, to measure the enzyme activity of APAO instead of SMO, use *N*¹-acetylspermine (Fluka Biochemika #01467; FW: 353.76 g/mol) as the substrate.

²0.083 M Glycine buffer may be stored at 4°C for several weeks exclusively for use in harvesting cells. However, for optimal results, the 0.5 M glycine to be used in the reaction mix should be made fresh on each assay date.

2.3. Common Laboratory Reagents and Disposables

1. Bradford protein assay reagent (Bio-Rad #500-0006).
2. Cell lifters (Fisher #08-773-1).
3. Dimethyl sulfoxide (DMSO; Sigma #D1435).
4. HPLC-grade water (J.T. Baker #4218-03).
5. Light-shielded (1.5, 15, 50 mL) and standard microcentrifuge tubes (1.5 mL).
6. Luminometer cuvettes (12 × 75 mm; BD Biosciences #556862).

3. Methods

3.1. Harvesting of Adherent Cells and Preparation of Cell Lysates

1. Remove culture media, rinse adherent cells with ice-cold 1× PBS, aspirate PBS.
2. Cover cells with 0.083 M glycine buffer, pH 8.0 (approximately 300 µL/well of 6-well dish or 700 µL/25 cm² flask), and place in −80°C freezer to lyse cells (see Note 5).
3. On day of assay, thaw flasks or dishes on ice, scrape cells with disposable cell lifters, transfer lysates to 1.5 mL tubes, and vortex 10s.
4. Centrifuge cell lysates (10 min, >12,000 × g, 4°C) and keep lysates on ice at all times during assay.

3.2. Prepare Assay Master Mix and Determine Hydrogen Peroxide Standard Curve

1. Preheat water bath containing a rack suitable for holding luminometer cuvettes to 37°C.
2. Thaw spermine, aminoguanidine, and pargyline on ice and thaw luminol at room temperature.
3. In a light-shielded conical tube on ice, prepare the assay reaction mix according to the chart in Table 1 for the desired number of reactions (see Notes 3 and ⁶) and mix by vortexing.
4. In light-shielded 15 mL tubes, serially dilute H₂O₂ in HPLC-grade water:

Dilution A: add 100 µL H₂O₂ (3% w/v solution, 0.882 M) to 8.72 mL HPLC-grade water to yield a 10 mM solution. Mix gently by inversion (see Note 7).

Dilution B: add 100 µL Dilution A to 10 mL HPLC-grade water to yield a 100 µM solution, and mix gently by inversion.

³Prepare the HRP solution only after all other components have been added to the assay reaction mix, immediately prior to proceeding with the assay protocol. Working quickly, dissolve HRP in HPLC-grade water to yield a 0.4 mg/mL solution, add the appropriate amount to the assay reaction mix on ice, and proceed with assay.

⁴While 30% w/v H₂O₂ solutions are available from chemical vendors (i.e., Sigma-Aldrich #216763), we have found 3% w/v solutions sold in supermarkets and drug stores to be preferable. These solutions are significantly less expensive, yield equivalent results, and can be replaced with a fresh bottle after several uses.

⁵Alternatively, following trypsinization of adherent cells, or for suspension cells, cells can be centrifuged, washed in ice-cold 1× PBS, resuspended in 0.083 M glycine, and then lysed at −80°C. In this case, thaw tubes containing cell lysates on ice and proceed with the protocol at step 3.1.4. Note: due to the presence of divalent metal cations in tissue homogenates, this assay protocol is not suitable for the measurement of oxidase activity in tissue samples.

⁶All samples should be assayed in triplicate; allow at least 18 reactions for the standard curve, 3 reactions for the blank, and three times the number of cell lysates to be assayed.

⁷Whenever working with H₂O₂ solutions, it is critical to work quickly and minimize the amount of time that the tubes containing H₂O₂ are open and exposed to air.

Dilution C: add 100 μL Dilution B to 10 mL HPLC-grade water to yield a 1 μM solution, and mix gently by inversion.

5. Prepare a series of solutions containing increasing concentrations of H_2O_2 (0–100 pmol/100 μL) for production of a standard curve relating H_2O_2 concentration to RLU, in a total volume of 300 μL :
 - 0 pmol: 1,000 μL HPLC-grade water.
 - 20 pmol: 800 μL HPLC-grade water + 200 μL Dilution C from step 3.2.4.
 - 40 pmol: 600 μL HPLC-grade water + 400 μL Dilution C.
 - 60 pmol: 400 μL HPLC-grade water + 600 μL Dilution C.
 - 80 pmol: 200 μL HPLC-grade water + 800 μL Dilution C.
 - 100 pmol: 1,000 μL Dilution C.
6. Pipette 200 μL reaction mix into a luminometer cuvette and incubate for 2 min in 37°C water bath.
7. Remove cuvette from water bath, remove condensation with Kimwipe, pipette 100 μL of the “0 pmol” solution into the reaction mix, and immediately read the luminescence for a 20 s integration time (see Note 8).
8. Repeat steps 3.2.6 and 3.2.7 to generate a standard curve with triplicate measurements of reactions containing 0, 20, 40, 60, 80, and 100 pmol H_2O_2 (18 total reactions) (see Note 9).
9. Using Microsoft Excel or other preferred method, fit a trend line to the standard curve data and calculate the r^2 value to validate the assay reaction mix (Fig. 2a) and for interpolation of raw sample RLU data.

3.3. Determination of SMO Enzyme Activity from Cell Lysates

1. If luminometer is equipped with an autoinjector, load and prime with 1.5 mM spermine substrate solution. Set the luminometer program to inject 50 μL /sample, followed by a 10 s delay and a 40 s integration of luminescence. Alternatively, the substrate can be manually pipetted into each cuvette as in step 3.2.3.
2. For blank, pipette 200 μL reaction mix into a luminometer cuvette, add 50 μL 0.083 M glycine buffer, pH 8.0, vortex briefly, and incubate 2 min in a 37°C water bath.
3. Remove cuvette from water bath, remove condensation with Kimwipe, and read luminescence as described in step 3.3.1.
4. Repeat steps 2 and 3 as necessary to measure blank and each cell lysate (50 μL supernatant from step 4) in triplicate. Alternatively, this protocol may be amended to assay enzyme kinetics of purified, recombinant SMO (see Note 10; Fig. 3).

⁸It is imperative to develop efficient and consistent timing and actions when adding the H_2O_2 to the reaction mix. For best results, we recommend placing the cuvette containing the 200 μL reaction mix into the luminometer, then pipetting the 100 μL H_2O_2 solution directly into the reaction mix (not down the side of the cuvette), and immediately starting the luminescence measurement. If this technique is followed, it is not necessary or advantageous to vortex the reaction mix + H_2O_2 mixture before reading.

⁹After becoming comfortable with the mechanics of the assay technique, it is possible to process samples (for both the standard curve and experimental cell lysates) more quickly using a staggered strategy. After starting the first 2-min incubation, wait about 45 s and then start preparing the second sample.

¹⁰For assay of purified SMO (or APAO) enzyme, the MAO and DAO inhibitors (pargyline and aminoguanidine) can be omitted and replaced with H_2O . Purified enzyme is diluted to 2 ng/ μL in 0.083 M glycine buffer, pH 8.0, then 50 μL is added to the reaction mix for each reaction, incubated 2 min at 37°C, substrate is added, and chemiluminescence is integrated over 20 s (16, 17).

5. Determine total protein concentration in each cell lysate supernatant using Bradford or other preferred method.
6. Calculate SMO activity as pmol H₂O₂ per min per mg protein, based on interpolation on the H₂O₂ standard curve (Fig. 2b).

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Fig. 1. Schematic illustrating the coupled enzyme reactions that comprise the enzyme activity assay described in this chapter. First, spermine oxidase (SMO) catalyzes the FAD-dependent oxidation of spermine to spermidine, 3-aminopropanal, and H₂O₂. Second, in the presence of this H₂O₂, luminol is oxidized by horseradish peroxidase (HRP), resulting in production of chemiluminescence.

Fig. 2.

(a) Representative plot of H_2O_2 standard curve displaying H_2O_2 concentration (0–100 pmol in 300 μL reaction; x -axis) versus 10^3 RLU/min (y -axis). A best-fit line is also plotted ($r^2 > 0.99$). (b) Representative data. A549 lung adenocarcinoma cells were treated for 24 h with or without 10 μM of the SMO-inducing polyamine analog, bis(ethyl)-norspermine (BENS). Cell lysate was harvested and SMO enzyme activity measured as described in this chapter.

Fig. 3. Data generated using alterations to the described protocol, as described in Note 10. Purified, recombinant spermine oxidase protein was used with the current protocol for the determination of kinetic properties. Plot depicts Lineweaver–Burk transformation ($r^2 > 0.99$) of SMO/PAOh1, assayed in the presence of increasing concentrations of spermine (0–250 μM).

Table 1

SMO enzyme activity assay setup and workflow

Reagent	Stock concentration	Final concentration	Per reaction (μL)	Per 100 reactions
Glycine, pH 8.0	0.5 M	0.083 M	50	5 mL
Aminoguanidine	100 mM	1 mM	3	300 μL
Pargyline	2.3 mg/mL	0.019 mg/mL	2.5	250 μL
Luminol	100 mM	0.017 mM	0.05	5 μL
HPLC-grade water			94.5	9.45 mL
Horseradish peroxidase	0.4 mg/mL	0.067 mg/mL	50	5 mL
Reaction mix			200	
Cell lysate or 0.083 M glycine blank			50	
<i>Vortex cuvette briefly and incubate for 2 min in 37°C water bath</i>				
Spermine	1.5 mM	0.25 mM	50	
Final reaction volume			300	
<i>Inject substrate and integrate luminescence for 40 s</i>				

The necessary assay reagents are indicated above as well as calculations for preparing a master mix for 100 reactions (see Subheading 2 for preparation instructions). The general assay scheme is also summarized (see Subheading 3 for detailed protocol)