ISSN : 0974 - 7435 Volume 9 Issue 9

BioTechnology
An Indian Journal

FULL PAPER

BTAIJ, 9(9), 2014 [376-382]

Seminal sulfhydryl oxidase: Appropriate fluorometric assay for the examination of spermatozoa disorders

Mahmoud Hussein Hadwan[1]*, Lamia A.Almashhedy[1], Abdul Razzaq S.Alsalman[2]
[1]Chemistry Dept., College of Science, Babylon University, (IRAQ)
[2]Surgery Dept., College of Medicine, Babylon University, (IRAQ)
E-mail : mahmoudhadwan@gmail.com

ABSTRACT

A Fluorometric assay for serum sulfhydryl oxidase activity that could be used as a back-up to current tests in the differential diagnosis of spermatozoa disorders is described. The assay is based on the H_2O_2-dependent oxidation of dichlorofluorescein catalyzed by peroxidase mimetic (a mixture of hematin (HT) and hemoglobin (HB)). The method is sensitive, precise (CV below 5.25%), and linear up to 200 μmol/l. The analytical recovery of the present method is estimated. The comparison with the colorimetric method gave good correlation (r = 0.9929). The method is applied to the measurement of the SHO activity in seminal plasma of fertile men and Asthenzoospermic patients. Reference values for seminal sulfhydryl oxidase activities determined with the present method on 60 healthy persons are 68.27 ± 4.03 nmol/mg protein.min.

KEYWORDS

Sulfhydryl oxidase;
Seminal plasma;
Fluorometric assay;
Spermatozoa disorders;
2', 7'-dichlorofluorescein.

INTRODUCTION

Study *in vitro* have established that sperm disulfide bonds can be reduced by sulfhydryl compounds in combination with detergents[2] or by action of sulfhydryl compounds alone (Lung, 1972). Sulfhydryl reduction generated in the de-condensation of sperm nuclei and in the loss of sperm tail structures. On the other hand, another study demonstrated that sperm disulfide bond reduction does not arise under normal physiological conditions[3], even with the presence in the reproductive tract of physiological sulfhydryl compounds[4]. A doable clarification for this inconsistency comes from the previous study of[5] who highlighted a unique enzyme thought to be concerned in controlling the levels of sulfhydryl compounds in the reproductive tract. This enzyme, a sulfhydryl oxidase, catalyzes the formation of *de novo* disulfide bonds between sulfhydryl groups with the following reduction of oxygen to hydrogen peroxide, as in equation below:

$$2R - SH + O_2 \rightarrow R - S - S - R + H_2O_2$$

Sulfhydryl oxidase enzyme is sometimes also indicated to thiol oxidase (EC 1.8.3.2), enzyme that also oxidize thiol groups using oxygen as electron acceptor but reducing it to water[6].

Previous assays for SHO employ one of the following principles. In the first, SHO activity is deter-

mined spectrophotometrically by monitoring the disappearance of thiols using discontinuous sampling with Ellman's reagent[7,17]. This assay undergoes certain disadvantages. It is sensitive, but cannot detect the exact levels of enzyme found in human seminal fluids in normal as well as under pathological conditions. Since it involves following small differences in a large background absorbance (particularly when K_m values for some thiol substrates are >5mM [1]).

The second assay utilizes the monitoring oxygen consumption polarographically. This method is convenient and widely utilized for the sulfhydryl oxidases[8,10]. This type of assays has some disadvantages such as the unavailability of laboratory equipment to perform in a large number of laboratories. Also, the disappearance of Oxygen is followed via a technique that is intrinsically not very sensitive[1] developed a method in which the peroxidase-mediated dimerization of homovanillic acid (HVA) by the action of hydrogen peroxide is monitored fluorometrically to measure SHO activity. This assay disregarded the interaction that results from the presence of the catalase enzyme in seminal fluids.

In this paper, precise fluorimetric method is introduced. The present assay has used 3-aminotriazol to eliminate the interaction with catalase. The assay is uncomplicated and the reagents used are relatively stable.

Principle

Sulfhydryl Oxidase catalyzes the formation of disulfide bridges at the expense of molecular oxygen:

$$2R-SH + O_2 \rightarrow R-S-S-R + H_2O_2$$

In the presence of H_2O_2, non-fluorescent DCFH (2', 7'-dichlorofluorescein) is oxidized to highly fluorescent 2', 7'-dichlorofluorescein (DCF; excitation, 495 nm; emission, 520 nm), which is detectable by vitreous fluorophotometry, as shown in Figure (1).

Reagents

A Hemoglobin 5* 10^{-7} M.

B Hematin stock solution is prepared by dissolving 0.05 g hematin in 25 ml 0.2 N sodium hydroxide. This solution remains stable for 3 months when refrigerated.

C 0.2 N sodium hydroxide: Dissolve 0.8 g of NaOH in 100mL of water.

D Hematin working solution is prepared by diluting 0.5-ml of hematin stock solution to 50 ml of 25 mM sodium phosphate buffer (pH 7.4).

E Peroxidase mimics solution is prepared by mixing equal volumes of Hemoglobin (HB) and Hematin (HT) working solution. (It is prepared immediately before using).

F Sodium phosphate buffer pH 7.4 (50 mM) is prepared by dissolving 1.1 g of Na_2HPO_4 and 0.27 g of KH_2PO_4 in 100 ml distilled water.

G 3-aminotriazole (50mM): is prepared by dissolving 0.42 gm of 3-aminotriazole in 100 ml of phosphate buffer (pH 7.4).

H DCFH was prepared from DCFH diacetate after the method described previously, 36 with some

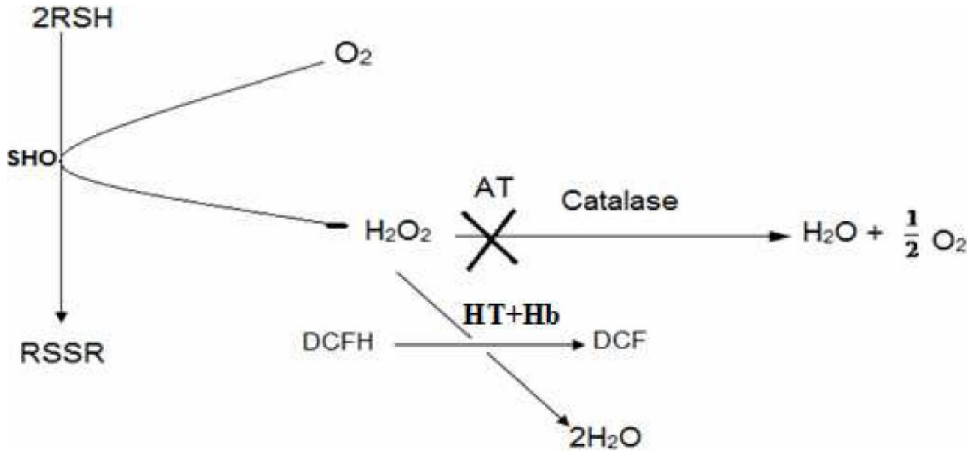

Figure 1 : The spectrophlorometric measurement of Sulfhydryl Oxidase (SHO). Hematin (HT) and hemoglobin (Hb) is added to catalyze the H_2O_2-dependent oxidation of DCFH to DCF, aminotriazole. 3-aminotriazole (AT) is included to inhibit interference by catalase.

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modifications. In brief, immediately before the fluorophotometric measurements, the hydrolysate of DCFH diacetate, 0.5 ml, was neutralized with 12 ml of 25 mM sodium phosphate buffer containing 20 µgm/ml hematin. The final concentrations of DCFH and hematin were 40 µM/ml and 19.2 µgm/ml, respectively.

I 300µM GSH: prepared by dissolving 0.1535 gm of a GSH in a final volume of 100 ml of 50 mM phosphate buffer (pH 7.5) solution (bulb with O_2)

J 200µM Hydrogen peroxide was freshly diluted and standardized daily using a molar extinction coefficient of 43.6 M^{-1} cm^{-1} at 240 nm.

Procedure

Pipette the following reagents into suitable vials, shown in TABLE 1:

Mix by vortex, after 5 min transfer the solutions to suitable cuvettes and read fluorescent intensity. Fluorescent intensity was linearly related to a DCF concentration between 1 nmol/1 and 2 µmol/1 with the fluorophotometer (DCF; excitation, 495 nm; emission, 520 nm). There is no need to use concentrated acids or high temperature to stop the reaction that catalyzed by a sulfhydryl oxidase, because reading is done by time course measurement (essential category found in spectrofluorometer shimadzu RF-5301), which helps to calculate the value of fluorescence after completing enzymatic reaction exactly.

Calculations

Standard curve

ΔI Standard = I Standard - I Standard blank

Plot the ΔI of the Standards vs. µmoles of H_2O_2.

Sample determination:

I Sample = I Test - I Test Blank (I: fluorcence intensity)

Determine the µmoles of H_2O_2 liberated using the Standard Curve.

$$\text{Units/L enzyme} = \frac{B}{(30)(0.03)} * df = (\mu mol/min)/L = (U/L)$$

a" mU/ml

B µmoles of H_2O_2 liberated according to standard curve.

df = Dilution factor.

30 = Time of assay in minutes.

0.03 = Volume (in milliliter) of enzyme used.

$$\text{m - Units/mg protein} = \frac{\text{m - units/ml enzyme}}{\text{mg protein/ml enzyme}}$$

Instrument

Spectrofluorometer shimadzu RF- 5301.

Statistical analysis

Statistical parameters such as: mean value (X), standard deviation (SD), and coefficient of variations (CV) are calculated with EXCEL.

RESULTS AND DISCUSSION

Although thiols such as glutathione interfere with the peroxidase-mediated determination of hydrogen peroxide, a very sensitive, continuous fluorescence assay of the sulfhydryl oxidase can be devised with appropriate selection of thiol substrate concentration and fluorogen. 2', 7'-Dichlorofluorescein (DCF) was found

TABLE 1 : Procedure

	Test	Test Blank	STD1	STD2	STD3	STD4	STD5	STD Blank
Sample	30 µL	-------	-------	-------	-------	-------	-------	-------
H_2O_2 STD	-------	-------	30 µL	50 µL	100 µL	200 µL	300 µL	-------
AT	500 µL	500 µL	500 µL	480 µL	430 µL	330 µL	230 µL	530 µL
Mix by inversion and equilibrate to 37°C for 5 min. Then add:								
GSH solution	400 µL	400 µL	400 µL	400 µL	400 µL	400 µL	400 µL	400 µL
Mix by inversion and incubate at 37C for exactly 25 minutes. Then add:								
DCFH	500 µL	500 µL	500 µL	500 µL	500 µL	500 µL	500 µL	500 µL
Peroxidase- mimics	250 µL	250 µL	250 µL	250 µL	250 µL	250 µL	250 µL	250 µL
* Sample	-------	30 µL	-------	-------	-------	-------	-------	-------

*** Serum is added to test blank to insure the prevention of overlapping of peroxides or $ONOO^-$ in sample (seminal plasma) with hydrogen peroxide that formed from the reaction, which catalyzed by sulfhydryl oxidase.**

to be a suitable fluorogen in the presence of 300μM thiols from reduced glutathione. Oxidation of non-fluorescent DCFH (dichlorofluorescein) to highly fluorescent 2', 7'-dichlorofluorescein (DCF; excitation, 495 nm; emission, 520 nm) by hydrogen peroxide with peroxidase as a catalyst is initially used to estimate hydrogen peroxide by[11]. After this scientific finding, several researchers focused studies on measuring hydrogen peroxide by using hemin, hematin, or cytochrome c as peroxidase mimics[12-15]. In the present assay, a new fluorometric method for assay sulfhydryl oxidase via hydrogen peroxide determination is presented, in which a mixture of hematin (HT) and hemoglobin (HB) is used as mimetic enzyme of peroxidase for fluorogenic reaction between 2', 7'-dichlorofluorescein and hydrogen peroxide. The obtained results demonstrated that the mixture of HB and HT is a promising peroxidase mimic. The emission spectrum of 2', 7'-dichlorofluorescein (DCF) is shown in Figure (2).

The concentration of 3-aminotriazole required to prevent interference with catalase that is founded in the sample, is investigated by assaying catalase activity in the presence of different concentrations of this inhibitor. Figure (3) indicates that the degree of catalase inactivation depends on the concentration and duration of pre-incubation with aminotriazole.

Aminotriazole (50mM) is included in all subsequent assays of SHO; this concentration is likely to be in excess of that necessary to inactivate the catalase present in seminal fluids. The increment of sulfhydryl oxidase activity after adding 50mM-aminotriazole reaches up to 40% and stays constant with higher concentration of this inhibitor. However, five min incubation with 50mM-aminotriazole allowed adding 100 k-unit of catalase (obtained from Himedia (Product Code: TC037)) to reaction mixture without any effect on the measured oxidase activity.

A crude sulfhydryl oxidase has been purified from chicken egg white according to method described by[8]. It is used to compare present method with others that are used for assessment of sulfhydryl oxidase. Results obtained by the present method are compared with those of colorimetric –method[17], as shown in TABLE (2). Identical samples, buffer, and substrate were used in both methods.

The method is applied to the measurement of the SHO activity in seminal plasma of 60 fertile men (age 31.6 ± 3.3 year) and 60 Asthenzoospermic patients (age 32.5 ± 3.23 year). The results elucidated in TABLE (3).

It is evident from the above that the results of this method can be used to assess oxidative stress of semi-

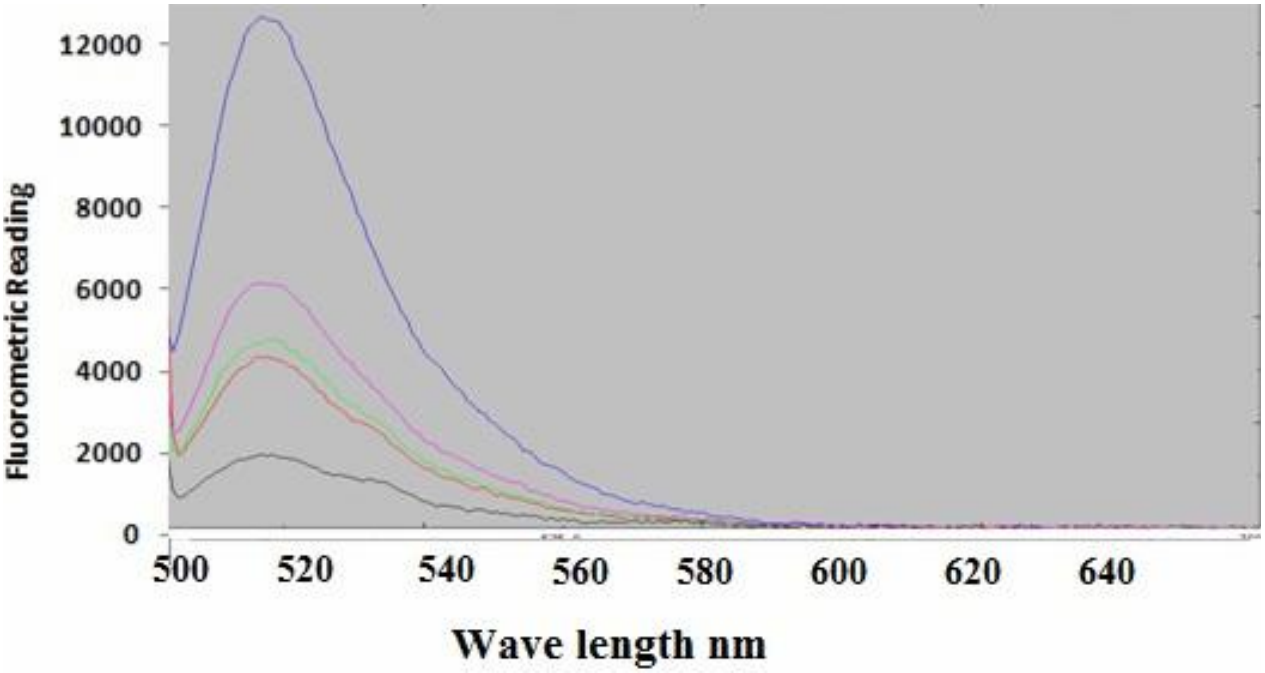

Figure 2 : Emission Spectrum of Reagent 2',7'-dichlorofluorescein (DCF). Excitation Spectra were Monitored at 495 nm, Emission Spectra were Monitored at 520 nm.

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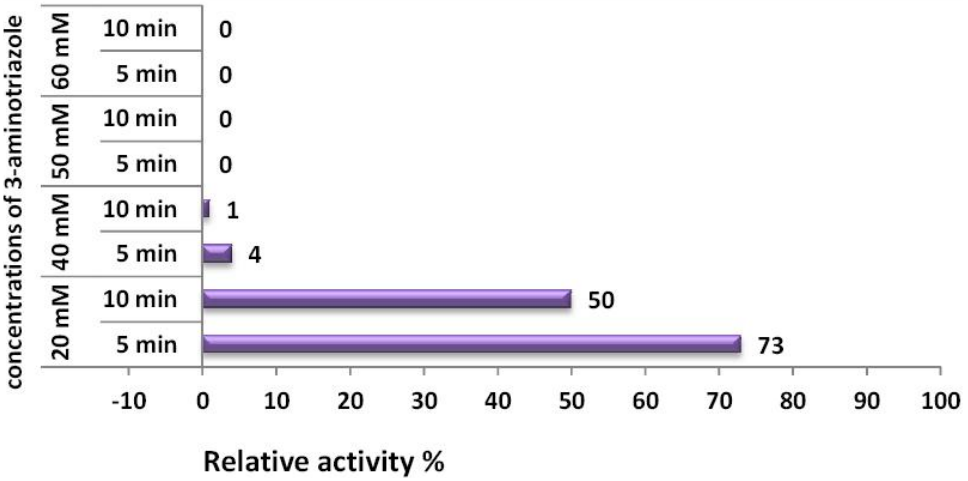

Figure 3 : The dependence of catalase activity (50 k-unit/L) on time of incubation with different concentrations of 3-aminotriazole.

TABLE 2 : Statistical Analysis of The Values Obtained for Sulfhydryl Oxidase by Colorimetric - Method and Present Method.

No. of Samples	20
Mean of colorimetric - method (nmol/ mg protein. min)	68.27 ± 3.1
Mean of test method (nmol/ mg protein. min)	68.41 ± 2.79
Mean of both methods (nmol/ mg protein. min)	86.34
Regression coefficient B	0.9839
Regression coefficient A	0.0161
Correlation coefficient	0.9929

TABLE 3 : Levels of sulfhydryl oxidase in seminal plasma of fertile and subfertile men.

	Healthy controls	Asthenzoospermic patients
Sulfhydryl oxidase (nmol/ mg protein. min) (mean± SD)	68.27 ± 4.03	56.15 ± 3.13

TABLE 4 : Analytical Recovery of Hydrogen Peroxide Added to Sample.

Present in assay	Equivalents of Hydrogen Peroxide	Calculated activity U/liter	Observed activity [a] µmol/liter	Recovery %
Pooled sample	-----	-----	71	-----
Pooled sample + peroxide [b]	10	81	81.443	99.99
Pooled sample + peroxide	20	91	89	98.9
Pooled sample + peroxide	100	171	169	98.83
Pooled sample + peroxide	200	271	263	98.52
Pooled sample + peroxide	1000	1071	1041	97.19

[a] **mean of triplicate determinations;** [b] **present as H_2O_2**

nal fluids.

Accuracy of the entire assay protocol is measured by recovery of hydrogen peroxide added to sample detailed in TABLE (4). Sample sulfhydryl oxidase purified from chicken egg white according to method described by[8].

The precision of the assay is measured in-run on a single sample specimen and between run over a period

TABLE 5 : The precision of the present assay

	N	Mean (±SD) µmol/liter	CV%
Within-run	20	68.4 ± 2.79	4.1
Between-run	20	66.9 ± 3.51	5.25

of three weeks with aliquots from the same sample pool but different reagent's preparations. The results are shown in TABLE (5).

The assay presents a number of advantages more than the existing methodologies. These advantages include; less quantity of sample is required (0.03 ml); the reagents are relatively stable; instrumentals, and apparatus are not complicated and available in most research laboratories, and the assay is free from interference.

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