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Fluorometric Assessment of Sulfhydryl Oxidase Activity: Optimization by Response Surface Methodology

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Abstract

Sulfhydryl oxidase was studied using a spectrofluorometric assay. The current protocol operates by using a combination of hemoglobin (HB) and hematin (HT) as a peroxidase mimic to catalyze the H₂O₂-dependent oxidation of thiamine. The response surface methodology is used to optimize the new method (RSM). The current method is very accurate, sensitive, and linear up to 200 IU. When compared to the colorimetric method, the method produced a satisfactory correlation. The novel protocol is being used to evaluate asthenospermic patients' and fertile men's seminal SHO activity. The current protocol was used to determine reference values for seminal sulfhydryl oxidase activity. Due to the fact the newly developed spectrofluorometric method is more sensitive and precise than other colorimetric methods, and because thiamine is less expensive than other types of probes used in colorimetric and spectrofluorometric methods, it is likely to find widespread use among scientists studying SHO activity in biological tissues. The present method's analytical recovery yielded high specific findings.

Keywords: Sulfhydryl Oxidase, Response surface methodology, Hematin, Hemoglobin, Spectrofluorometric Assessment.

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Graphical abstract:

Introduction

The plasma thiol pool is principally composed of protein thiols, albumin thiols and slightly composed of thiols that have low-molecular-weight such as cysteine (Cys), homocysteine, γ -glutamylcysteine, cysteinylglycine and glutathione, [1]. Oxidation reactions always act to convert the reduced form of thiols (RSH) to the oxidized form (RSSR) via creating the disulphide bonds [2]. A disulphide bond has a covalent bond property and is also called a disulphide bridge or SS-bond. Under disorders of oxidative stress inclination, the oxidation process of Cys residues can form the reversible production of integrated disulphide bridges between low-molecular-mass thiols and protein thiol groups. Dynamic thiol–disulphide homeostasis is maintained via reduction of the formed disulphide bonds again to reduced thiol groups [3]. The status of dynamic thiol disulphide homeostasis has critical roles in detoxification, antioxidant protection, apoptosis, signal transduction, regulation of enzymatic activity and cellular signaling mechanisms and transcription factors [4]. Additionally, the homeostasis of dynamic thiol disulphide is progressively involved in many disorders. Several scientific reports indicate that an atypical thiol disulphide homeostasis state is implicated in the pathogenesis of some types of infertility such as asthenospermia [5].

The most important explanation for the thiol disulphide homeostasis comes from Chang and Morton's [6] study who discovered an exceptional enzyme thought to be involved in regulating the concentrations of sulfhydryl groups in the reproductive tract. A sulfhydryl oxidase acts to catalyze the production of disulfide bonds between sulfhydryl groups with the formation of hydrogen peroxide, as in the equation below:

Sulfhydryl oxidases include flavin adenine dinucleotide (FAD) as a prosthetic group. These enzymes have been recognized in animals [7], and plants [8]. Thiol oxidase is another enzyme concerned with the thiol disulphide homeostasis, this enzyme oxidizes thiol groups using oxygen as an electron acceptor, reducing it to water [9]. SHO acts to form the *de novo* disulfide bonds as a result, to reduce SH groups via enzymatic reduction of oxygen to form hydrogen peroxide. Moderate thiol levels are a necessary factor to maintain the defensive function and sperm motility and metabolism of spermatozoa under *in vitro* conditions [10].

The subsequent principles were utilized in the previous assays for SHO. The first utilize Ellman's reagent spectrophotometrically to monitor the disappearance of thiols by using discontinuous sampling [11, 12]. The disadvantages of this assay includes it is usability to detect the precise activity of SHO that is found in human seminal fluids, because it depends on monitoring small changes in a large background absorbance. Principally, the K_m values for some thiol substrates reach more than 5mM [13].

The monitoring of oxygen consumption with polarography represents the second assay. This method is suitable and commonly applied for the estimation of

sulfhydryl oxidase activity [14, 15]. These methods have some disadvantages such as the absence of laboratory apparatus to achieve SHO activity in a large number of laboratories. Additionally, the monitoring of oxygen disappearance is achieved via an instrument that is fundamentally not very sensitive [13].

Raje *et al.*, [13] established fluorometrical assay to measure SHO activity, which depends upon the dimerization of homovanillic acid (HVA) that is catalyzed by the peroxidase enzyme in the presence of hydrogen peroxide. This assay discounted the interference that results from the existence of the catalase enzyme in seminal fluids.

A new spectrofluorometric method was developed by utilizing the H₂O₂-dependent oxidation of dichlorofluorescein that catalyzed by peroxidase mimetic (a mixture of hematin (HT) and hemoglobin (HB)) [16].

This paper describes a simple and accurate spectrofluorometric method. The assay is used peroxidase mimetic (a mixture of hematin (HT) and hemoglobin (HB)) to monitor the fluorometric H₂O₂-dependent oxidation of thiamine. Response surface methodology (RSM) was applied as a tool to optimize the new method.

Principle

SHO catalyzes the oxidation of reduced thiol to form disulfide bonds in the presence of molecular oxygen:

The assay is used peroxidase mimetic (a mixture of hematin (HT) and hemoglobin (HB)) to monitor the fluorometric H₂O₂-dependent oxidation of thiamine. The end product is called thiochrome, which has fluorescence property (at Ex/Em = 370/425 nm). Aminotriazole (AT) is added to enzymatic reaction to inhibit catalase enzyme and prevents the consumption of hydrogen peroxide, as shown in fig (1). Since the intensity of fluorescence is directly proportional to SHO level; the SHO activity can be accurately measured.

Figure 1. The details of the fluorometric assessment of sulfhydryl oxidase (SHO). peroxidase mimetic (a mixture of hematin (HT) and hemoglobin (HB)) to monitor the fluorometric H_2O_2 -dependent oxidation of thiamine. 3-aminotriazole (AT) is involved in enzymatic reaction to eliminate the interference with catalase enzyme.

Reagents

- Sodium hydroxide (0.2 N) is prepared by dissolving 0.8 g of NaOH in 100mL of distilled water.
- Hematin stock solution is composed of 0.05 g hematin that dissolved in 25 ml of 0.2 N sodium hydroxide.
- Hematin working solution is composed of 0.5 ml of hematin stock solution that diluted to 50 ml of 25 mM sodium phosphate buffer (pH 7.4).
- Hemoglobin 5×10^{-7} M.
- Peroxidase mimics solution is consisted of equal volumes of Hematin (HT) working solution and Hemoglobin (HB).
- A phosphate buffer (100 mM; pH 7.0) was prepared by mixing solutions a and b at a ratio of 1:1.5. Solution (a) was prepared by dissolving 13.62 g of KH_2PO_4 in one liter of distilled water, and solution (b) was prepared by dissolving a 17.9 g of $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ in one liter of distilled water.
- 3-aminotriazole (AT) (50mM): is consisted of 0.42 gm of 3-aminotriazole that is dissolved in 100 ml of phosphate buffer solution (pH 7.4).
- Thiamin stock solution (10 mM) is composed of 337 mg of thiamine hydrochloride that is dissolved in 100 ml of water. Refrigerated at 4°C .
- Thiamin working solution (2.5×10^{-3} M) is composed of one volume of stock solution that is dissolved to four volumes with distilled water.
- Glutathione solution (300 μM) is composed of 0.1535 gm of a GSH that is dissolved in a final volume of 100 ml of 50 mM phosphate buffer (pH 7.5) solution (bulb with O_2).
- Hydrogen peroxide (200 μM) was prepared by suitable diluting of standard hydrogen peroxide (30%) using a molar extinction coefficient of $43.6 \text{ M}^{-1} \text{ cm}^{-1}$ at 240 nm. This solution was freshly prepared and standardized daily.

Procedure

Enzyme activity was measured according to the procedure described in Table 1:

Table 1. The details of the procedure that used for measuring the SHO enzyme activity.

	Test	Test Blank	STD1	STD2	STD3	STD4	STD5	STD Blank
Sample	30 μ L	-----	-----	-----	-----	-----	-----	-----
H ₂ O ₂ STD	-----	-----	30 μ L	50 μ L	100 μ L	200 μ L	300 μ L	-----
AT	500 μ L	500 μ L	500 μ L	480 μ L	430 μ L	330 μ L	230 μ L	530 μ L
Mix by inversion and equilibrate to 37°C for 5 min. Then add:								
GSH solution	400 μ L	400 μ L	400 μ L	400 μ L	400 μ L	400 μ L	400 μ L	400 μ L
Mix by vortex and incubate for 10 minutes at 37°C, after that, the reaction was terminated with 0.5 ml of 8% TCA								
Mix well and centrifuge for 15 minutes at 3000 xg, then remove 0.5 ml of supernatant in a clean tube , and add:								
Sodium hydroxide	500 μ L	500 μ L	500 μ L	500 μ L	500 μ L	500 μ L	500 μ L	500 μ L
Thiamine	500 μ L	500 μ L	500 μ L	500 μ L	500 μ L	500 μ L	500 μ L	500 μ L
Peroxidase-mimics	250 μ L	250 μ L	250 μ L	250 μ L	250 μ L	250 μ L	250 μ L	250 μ L
* Sample	-----	30 μ L	-----	-----	-----	-----	-----	-----
All the test tubes were mixed and leaved for 10 min at room temperature. All the solutions were transferred to suitable cuvettes and the fluorescent intensity were read (excitation, 370 nm; emission, 425 nm).								

* Sample is added to test blank tube to omit the interference that arises from the presence of peroxides or peroxynitrate in semen samples with hydrogen peroxide that formed from the sulfhydryl oxidase catalyzed reaction.

$$\Delta I \text{ Standard} = I \text{ Standard} - I \text{ Standard blank (I: intensity of fluorescence)}$$

Plot the ΔI of the Standards vs. μ moles of H₂O₂.

Sample Determination:

$$I \text{ Sample} = I \text{ Test} - I \text{ Test Blank (I: intensity of fluorescence)}$$

Determine the μ moles of H₂O₂ liberated using the Standard Curve.

$$\text{Enzyme activity Unit/L (mU/ml)} = \frac{B}{10 * 0.03} * df$$

Where:

B μ moles of H₂O₂ liberated according to standard curve.

df = Dilution factor.

10 = Time of assay in minutes.

0.03 = Volume (in milliliter) of enzyme used.

Instrument

Spectrofluorometer shimadzu RF- 5301.

Optimization of the current method

The concentration of 3-aminotriazole that was required to prevent the interference with catalase enzyme was investigated by utilizing RSM and using a Box-Behnken design [17, 18]. The statistical parameters were estimated using the Design Expert Software (DES), Version 8.0.6, Stat-Ease Inc, USA. The enzymatic reaction of catalase reaction using (50 k-unit/L) was performed with different concentrations of 3-aminotriazole and different periods of incubation time. The volume of reaction was adjusted to 1mL and the catalase activity was calibrated using the peroxovanadate method as described by Hadwan and Ali [19]. The concentration of 3-aminotriazole, suitable time of incubation and thiamin concentration were carried out as independent variables (Table 1), and the sulfhydryl oxidase activity of the standard enzyme (30 IU) as a dependent variable. The second order polynomial equation was applied for arithmetic modeling to get the correlation between the independent and dependent variables (Eq. 1). The analysis and design of the RSM experiment were carried out using the software of Design Expert 12.

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j + \varepsilon \quad (1)$$

Where β_0 represent the intercept, β_i represents the linear coefficient, β_{ii} represents the quadratic coefficient and β_{ij} interaction coefficients. The residual is designed as ε .

Table 2: Box-Behnken was designed to determine the optimal condition of the sulfhydryl oxidase activity. The 3-aminotriazole concentration, suitable time of incubation and thiamin concentration are also involved as independent variables as shown in Table 1, and the sulfhydryl activity of the standard enzyme (30 IU) as a dependent variable. The values were expressed as mean $\pm$ SD (n=3).

Run ^a	Independent variables			Dependent variable
	3-Aminotriazole (mM)	Incubation Period (min)	Thiamin Concentration (mM)	Sulfhydryl Oxidase Activity
1	55	10	2.5	30
2	55	20	1	3
3	10	20	1.5	3
4	100	1	5.5	10
5	10	5.5	1	10
6	100	10	5.5	29
7	55	5.5	5.5	28
8	55	1	10	2
9	100	5.5	10	29
10	55	5.5	5.5	28
11	10	1	5.5	2
12	55	10	5.5	28
13	100	5.5	1	4
14	55	5.5	5.5	28
15	10	5.5	10	3
16	55	1	1	3
17	55	5.5	5.5	28

^a Random experiments creating by use Box-Behnken design.

Statistical analysis

Data analysis was achieved using SPSS 24 for Windows (SPSS Inc., Chicago, IL, USA). Data were expressed as mean, standard deviation (SD), and coefficient of variations (CV).

Results and discussion

Although thiol compounds such as cysteine and glutathione change the precision of the peroxidase-mediated assessment of hydrogen peroxide concentration, a sensitive and precise fluorescence assay of the sulfhydryl oxidase can be developed. The method depends upon the formation of highly fluorescent thiochrome (excitation, 370 nm; emission, 425 nm) as a result of the oxidation of non-fluorescent thiamine by hydrogen peroxide/peroxidase system. Thiamine was established to be an appropriate fluorogen in the enzymatic reaction that contains 300 μ M reduced thiol compounds [16]. The formation of fluorescent thiochrome from the oxidation of non-fluorescent thiamine via the peroxide/ peroxidase or peroxide/peroxidase mimic system is frequently used to estimate thymine [20, 21] or to measure hydrogen peroxide [22].

Previous methods have used hemin, metal-porphyrin complex [23], hematin [24] or hemoglobin [22] as peroxidase mimics. The current method developed a new fluorometric assessment for sulfhydryl oxidase activity via hydrogen peroxide determination. The method utilizes a mixture of hematin (HT) and hemoglobin (HB) as mimetic peroxidase for stoichiometric reaction between thiamin and hydrogen peroxide. The results of the present study confirmed that the mixture of HT and HB is a good peroxidase mimic. The spectroscopic spectra of thiamine are shown in Figure 2.

Figure 2: Emission spectra produced thiochrome. excitation spectra at 370 nm and emission spectra at 425 nm.

The selective inhibitor of catalase (3-aminotriazole) was used to eliminate the expected interference which may arise from the existence of such an enzyme.

Optimization of the current method

In order to attain the best conditions, statistical processes were performed to the BBD [17, 18]. The ANOVA analysis for the second order polynomial equations and results for the linear coefficients, quadratic coefficients and interaction coefficients are listed in Table 3. Statistical analysis shows that the postulated model has a significant fit of results. The resultant F-value (47.94) showed that the model was significant. The model terms were significant ($p = 0.0001$), according to additional findings. As a result, the SHO assay's ANOVA revealed that the actual correlation between the three independent variables of the proposed model was both appropriate for representation and highly significant. In order to examine the interaction effects of the three independent variables, contour graphs and three dimensional (3D) in the BBD were used. When the third component remained constant, the creation of the graphs at the midway level was reliant on a combination of two variables.

Figure 2 (a and b) shows the contour map of the impact of the independent variables on sulfhydryl oxidase activity. The results show a dependency on the 3-aminotriazol concentration, where it is found to be linear with ($p < 0.001$) and quadratic with ($p < 0.05$) effects and significantly affected by thiamine concentration. Other

factors which participate in clarity involved the linear effect with ($p < 0.001$) and quadratic effect with ($p < 0.01$) of time.

Figure 2-a: Response surface for activity of sulfhydryl oxidase as a function of 3-aminotriazole concentration (mM) and time (min) (at 37°C).

Figure 2-b: The contour plots of sulfhydryl oxidase activity as a function of the concentration of 3-Aminotriazole (mM) and time (min) (at 37°C).

Table 3: The ANOVA for quadratic studying model.

Source	Sum of Squares	df	Mean Square	f-value	p-value	
Model	2496.56	9	277.40	47.94	< 0.0001	significant
A.3-aminotriazole Conc,	364.50	1	364.50	63.00	< 0.0001	
B. Time	288.00	1	288.00	49.78	0.0002	
C. Thiamin Conc.	242.00	1	242.00	41.83	0.0003	
AB	81.00	1	81.00	14.00	0.0072	
AC	256.00	1	256.00	44.25	0.0003	
BC	196.00	1	196.00	33.88	0.0006	
A ²	236.84	1	236.84	40.94	0.0004	
B ²	380.00	1	380.00	65.68	< 0.0001	
C ²	341.05	1	341.05	58.95	0.0001	
Residual	40.50	7	5.79			
Pure Error	0.0000	4	0.0000			
Lack of Fit	40.50	3	13.50			
Cor Total	2537.06	16				

Factor coding is **Coded**.

Summation of squares is **Type III - Partial**

The **F-value of Model** is 47.94 reveal that it's a significant model. This mean that only a 0.01% chance of F-value to be due to noise.

P-values < 0.05 mean that the terms of model are significant. In present state A, B, C, AB, AC, BC, A², B², C² found to be a significant terms of model. Values > 0.1000 mean that the terms of model are insignificant. If the terms of model are insignificant, the reduction of model may improve it.

The greatest SHO activity was achieved under optimal conditions, which were 55 mmol L⁻¹ concentration of 3-aminotriazole, 10 min suitable time of incubation, and 2.5 mmol L⁻¹ thiamin concentration. The highest activity was at 30 U/L. The actual value was principally compatible with the predicted value, showing that the RSM analysis was consistent and fitted well with the practical conditions in the laboratory.

Several researchers focused studies on measuring hydrogen peroxide by using hemin, hematin, or cytochrome c as peroxidase mimics [25-28]. In the present assay, a new fluorometric method for assay sulfhydryl oxidase via H₂O₂ estimation is presented, that contains a mixture of hematin (HT) and hemoglobin (HB) used for mimetics peroxidase enzyme in fluorogenic reaction between thiamine and H₂O₂. Results obtained in the present study demonstrated that the mix of HB and HT is a promising peroxidase mimic.

Aminotriazole (50mM) is included in all subsequent assays of SHO; this concentration is likely to be more than 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 a higher concentration of this inhibitor. However, five minutes' incubation with 50mM-aminotriazole allowed for

adding one hundred k-units of catalase [obtained from Himedia (Product Code: TC037)] to the reaction mixture without any effect on the measured oxidase activity.

Accuracy, reproducibility, selectivity and of the protocol

A crude SHO enzyme was purified from hen egg white according to a method described by Hooper *et al.*, [14]. It is used to compare the current method with other methods used to determine SHO activity. The results of the present study were compared with the previous colorimetric one [12], as listed in Table 4. The same specimens, substrate and buffer were utilized in two methods.

Table 4: Comparison between fluorometric and colorimetric method

Specimens Numbers	20
Mean of fluorometric method (nmol/ mg protein. min)	31.41 ± 3.55
Mean of colorimetric method (nmol/ mg protein. min)	30.88 ± 2.2
Regression coefficient A	0.0115
Regression coefficient B	0.9921
Correlation coefficient	0.9911

Method application

The present fluorometric method was applied to measure SHO activity in plasma of seminal fluid of 60 healthy fertile men with mean age of (33.5 ± 5.5 years) and 60 asthenzoospermic men with a mean age of (35.5 ± 4.5 years). The results are elucidated in Table 5.

Table 5: Sulfhydryl Oxidase activities in plasma of seminal fluid of healthy fertile and asthenzoospermic men.

	Healthy controls	Asthenzoospermic patients
Sulfhydryl oxidase (nmol/ mg protein. min) (mean± SD)	70.82 ± 4.03	55.5 ± 8.6

It is evident from the above that the results of this method can be used to assess the oxidative stress of seminal fluids.

Accuracy and precision of the protocol

The accuracy of the whole method was determined by the recovery of H₂O₂ added to specimens which is detailed in Table 6.

Table 6: The recovery of H₂O₂

Present in assay	Equivalents of H ₂ O ₂	Calculated Activity U/liter	Observed activity ^a μmol/liter	Recovery %
Pooled sample	-----	-----	30	-----
Pooled sample + peroxide ^b	10	40	38	95
Pooled sample + peroxide	20	50	51	98
Pooled sample + peroxide	100	130	132	98.4
Pooled sample + peroxide	200	230	221	95.5
^a mean of triplicate assay				
^b present as H ₂ O ₂				

The precision of the assay is measured in-run on a single sample specimen and run over a period of three weeks with aliquots from the same sample pool but different reagent's preparations. The results are shown in Table 7.

Table 7: The precision of fluorometric method for determination of SHO activity

	N	Mean (±SD) μmol/l	CV%
Within-run	20	30.88 ± 1.2	3.8
Between-run	20	28.8 ± 1.5	5

The newly developed method presents many advantages over other methods to determine SHO activity includes; the small volume of the sample, relatively stable reagents of the method, simple handling with instrument and interference-free assay.

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Data availability

All the data are available.

Ethics approval and consent to participate.

Ethics Committee (University of Babylon/ College of Science/ Iraq), Ref. no.: 3155
Date: 11/9/ 2019.

Conflicts of Interest

The authors declare no conflicts of interest or competing interests.

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Consent for publication

The authors give their consent for the publication of identifiable details, which can include photograph(s) and/or videos and/or case history and/or details within the text (“Material”) to be published in the Fluorescence Journal and Article. Therefore, anyone can read material published in the Journal.

Authors' contributions

The authors confirm contribution to the paper as follows: study conception and design: Mahmoud Hadwan; data collection: Abdul Razzaq S. Als Salman, Lamia A. Almashhedy, Abdulsamie H. Alta'ee, Mahmoud H. Hadwan, Asad M. Hadwan; analysis and interpretation of results: Abdul Razzaq S. Als Salman, Lamia A. Almashhedy, Abdulsamie H. Alta'ee, Mahmoud H. Hadwan; draft manuscript preparation: Abdul Razzaq S. Als Salman, Lamia A. Almashhedy, Abdulsamie H. Alta'ee, Mahmoud H. Hadwan, Asad M. Hadwan. All authors reviewed the results and approved the final version of the manuscript.

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