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 (RSM) is used to optimize the new method. 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 sulfhydryl oxidase 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 sulfhydryl oxidase activity in biological tissues. The present method's analytical recovery yielded high specific findings.

Keywords Sulfhydryl Oxidase · Response surface methodology · Hematin · Hemoglobin · Spectrofluorometric Assessment

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, antioxidation 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

Highlights
• A novel technique for detecting the activity of the sulfhydryl oxidase enzyme in biological samples was investigated
• The spectrofluorometric method was applied
• The validation procedure demonstrated that the diagnostic technique works with biological material
• The protocol may be used as a diagnostic tool and is appropriate for research purposes

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groups in the reproductive tract. A sulfhydryl oxidase (SHO) acts to catalyze the production of disulfide bonds between sulfhydryl groups with the formation of hydrogen peroxide, as in the equation below:

\[2R - SH + O_2 \rightarrow R - S - S - R + H_2O_2\]

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]. Sulfhydryl oxidase acts to form the de novo disulfide bonds as a result, to reduce sulfhydryl (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]. This protocol fails to measure sulfhydryl oxidase activity in seminal fluids, because it depends on monitoring small changes of sulfhydryl group (–SH) concentration in a large background absorbance. Principally, the \(K_m\) values for some thiol substrates reach more than 5 mM [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 \(H_2O_2\)-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 \(H_2O_2\)-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:

\[2R - SH + O_2 \rightarrow R - S - S - R + H_2O_2\]

The assay is used peroxidase mimetic (a mixture of hematin (HT) and hemoglobin (HB)) to monitor the fluorometric \(H_2O_2\)-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 Scheme (1). Since the intensity of fluorescence is directly proportional to SHO level; the SHO activity can be accurately measured. Hydrogen peroxide concentration was adjusted using a molar extinction coefficient of 43.6 M⁻¹ cm⁻¹ at 240 nm [19].

**Reagents, Procedure and the Details of the Optimization of the Current Method**

Sections are Elucidated in the Supplementary Information

**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 µM 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 sulphhydryl oxidase activity via hydrogen peroxide determination. The method utilizes a mixture of hematin (HT) and hemoglobin (HB) as mimetic peroxidase for stoichiometric reaction of thiamine. 3-aminotriazole (AT) is involved in enzymatic reaction to eliminate the interference with catalase enzyme.

Scheme 1. The details of the fluorometric assessment of sulphhydryl oxidase (SHO), peroxidase mimic (a mixture of hematin (HT) and hemoglobin (HB)) to monitor the fluorometric \( \text{H}_2\text{O}_2 \)-dependent oxidation of thiamine. 3-aminotriazole (AT) is involved in enzymatic reaction to eliminate the interference with catalase enzyme.

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

**Fig. 1** Emission spectra produced thiochrome. Excitation spectra at 370 nm and emission spectra at 425 nm. Sulphhydryl oxidase activity correlated with the spectrophotometric properties of the produced thiochrome compound. The fluorescence spectra achieved for the resulting thiochrome compound are: a 40 \( \mu \text{M} \) thiochrome; b 35 \( \mu \text{M} \) thiochrome; c 30 \( \mu \text{M} \) thiochrome; d 25 \( \mu \text{M} \) thiochrome; e 20 \( \mu \text{M} \) thiochrome and f 15 \( \mu \text{M} \) thiochrome.
Optimum Concentrations of the Reagents of the Current Method

In order to attain the best conditions, statistical processes were performed to the Box–Behnken design (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 1. 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. Also, the model terms were significant according to the obtained p-value (p = 0.0001). 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 2a, 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.

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 (50 mM) 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

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Table 1 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 |
| 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  |
| A²                    | 236.84        | 1   | 236.84      | 40.94   | 0.0004  |
| B²                    | 380.00        | 1   | 380.00      | 65.68   | <0.0001 |
| C²                    | 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
a  Factor coding: Actual Design Points:

- Above Surface
- Below Surface

X1 = A: 3-Aminotriazole Concentration
x2 = B: Time

Actual Factor
C: Thiamine Concentration = 5.5

b  Factor coding: Actual Design Points:

- Design Points

X1 = A: 3-Aminotriazole Concentration
x2 = B: Time

Actual Factor
C: Thiamine Concentration = 5.5
fluids. The increment of sulphhydryl oxidase activity after adding 50 mM-aminotriazole reaches up to 40% and stays constant with a higher concentration of this inhibitor. However, five minutes’ incubation with 50 mM-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 and Selectivity of the Protocol**

A crude SHO enzyme was purified from hen egg white according to a method described by Hoober 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 2. The same specimens, substrate and buffer were utilized in two methods.

**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 asthenozoospermic men with a mean age of (35.5 ± 4.5 years). The results are elucidated in Table 3.

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

**Precision of the Protocol**

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 4.

The previous method that described in ref. (16) was utilized dichlorofluorescein that catalyzed by peroxidase mimetic (a mixture of hematin (HT) and hemoglobin (HB) to assess the produced \( \text{H}_2\text{O}_2 \), as a result to SHO enzymatic activity. The current method was utilized thiamin instead of 2′,7′-dichlorofluorescein to complete the SHO enzymatic activity. The current protocol is preferred over the previous method for several reasons. Firstly, 2′,7′-dichlorofluorescein is not selective probe for hydrogen peroxide and used to assess concentration of total reactive species [29]. Also, the superoxide disproportionation forms hydrogen peroxide which, in the presence of peroxidase activity, will oxidize more dichlorofluorescein-reduced form (DCFH) to dichlorofluorescein-oxidized form (DCF) with self-amplification of the fluorescence. Moreover, esterases enzyme can produce hydrogen peroxide by the deacetylation of dichlorofluorescein-diacetate (DCFH-DA). That is mean the use of this probe to measure hydrogen peroxide formation in cells is problematic [30]. In the same context, dichlorofluorescein interferes with glutathione, NADH and ascorbic acid [31]. On the other hand, thiamine is most commonly quantified following oxidation by hydrogen peroxide to the blue fluorescent product thiochrome. This forms the basis for quantification in the commonly used biochemical protocols [32–34].

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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**Authors’ Contributions** The authors confirm contribution to the paper as follows: study conception and design: Mahmoud Hadwan; data collection: Abdul Razzaq S. Alsalman, Lamia A. Almashhedy, Abdulsamie H. Alta’ee, Mahmoud H. Hadwan, Asad M. Hadwan; analysis and interpretation of results: Abdul Razzaq S. Alsalman, Lamia A. Almashhedy, Abdulsamie H. Alta’ee, Mahmoud H. Hadwan; draft manuscript preparation: Abdul Razzaq S. Alsalman, Lamia A.

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**Table 2** 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 |

**Table 3** Sulphhydryl Oxidase activities in plasma of seminal fluid of healthy fertile and asthenozoospermic men

| Specimens | Healthy controls | Asthenozoospermic patients |
|-----------|-----------------|---------------------------|
| Sulphhydryl oxidase (nmol/ mg protein. min) (mean ± SD) | 70.82 ± 4.03 | 55.5 ± 8.6 |

**Table 4** 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 |
Almashhedy, Abdulsamie H. Alta’ee, Mahmoud H. Hadwan, Asad M. Hadwan. All authors reviewed the results and approved the final version of the manuscript.

**Data Availability** All the data are available.

**Declarations**

**Ethics Approval and Consent to Participate** Ethics Committee (University of Babylon/ College of Science/ Iraq), Ref. no.: 3155 Date: 11/9/2019.

**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.

**Conflicts of Interest** The authors declare no conflicts of interest or competing interests.

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