

A Sensitive, Accurate, and Versatile Method for the Quantification of Superoxide Dismutase Activities in Biological Preparations

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ABSTRACT | Superoxide dismutase (SOD) plays a major role in antioxidant defense and redox regulation in eukaryotic cells and whole organisms. We describe here a sensitive and reliable method to characterize different SOD activities in a variety of biological samples, ranging from budding yeast to human cerebrospinal fluid. It is the spectrophotometric assay developed by Marklund and Marklund in 1974, based on the inhibition of pyrogallol autoxidation, which we have optimized for different isoenzymes, cell types, tissues, pH, buffers, and temperatures. By adjusting the assay conditions to multi-well plate readers, we show here that the method is suitable for the analyses of SOD activity in a number of samples and conditions. The procedure involves inexpensive reagents, and allows for a rapid, sensitive, versatile, and reproducible measurement of SOD activity in a wide variety of biological samples.

KEYWORDS | Pyrogallol autoxidation; Spectrophotometric methods; Superoxide dismutase

ABBREVIATIONS | CSF, cerebrospinal fluid; DTPA, diethylenetriaminepentaacetic acid; ECSOD, extracellular tetrameric superoxide dismutase; NBT, nitroblue tetrazolium; PRP, platelet-rich plasma; ROS, reactive oxygen species; SOD, superoxide dismutase; UV, ultraviolet

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1. OVERVIEW

Reactive oxygen species (ROS) are produced as by-products in aerobic metabolic processes. ROS include, among others, species such as superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^{\bullet}), which are capable of reacting with various intracellular targets, eventually leading to oxidative damage of cellular lipids, proteins, and nucleic acids [1–3]. Although ROS were initially considered the main culprits of oxidative cell death, recent studies have demonstrated that ROS are also necessary for proper cell functioning, including regulation of cell proliferation, cell differentiation [4], and behave as signaling molecules of physiological responses in redox-sensitive signal transduction pathways [5].

Under physiological conditions, a neutral balance between levels of ROS and antioxidants favors cellular homeostasis. Disrupting this balance, either through an increased production of ROS or decreased levels of antioxidants, provokes a condition referred to as oxidative stress [6]. To prevent or repair the damage caused by ROS, cells contain several defenses that include enzymatic and no-enzymatic antioxidants. One of the most important antioxidant enzymes is superoxide dismutase (SOD). SOD cata-

lyzes the conversion of superoxide to H_2O_2 and molecular oxygen (O_2). Eukaryotic organisms possess three isoforms of SOD enzymes, which are highly compartmentalized: a cytosolic copper-zinc dimeric form, known as Cu,ZnSOD or SOD₁; a mitochondrial tetrameric manganese superoxide dismutase MnSOD or SOD₂ [7]; and an extracellular tetrameric superoxide dismutase (ECSOD) or SOD₃ [8].

Different methods for the determination of SOD activity have been published [9–11]. Each method differs in terms of experimental conditions, including reagents, reaction conditions, media composition, and also by the analytical methods used to monitor enzyme activity. These have been classified as positive and negative assays [12]. Positive spectrophotometric assays proposed for determination of SOD activity are not as specific as in the analysis of cell homogenates or cellular fractions, requiring the complete elimination of catalase activities in the samples before assay [13]. Negative spectrophotometric assays have many advantages: the superoxide decay curve is directly observed, and generally the equipment required is simple and available in any laboratory. Usually, negative assays are based on the inhibition of oxidation process of different compounds by SOD. The most commonly used negative test technique (included in different commercial kits)

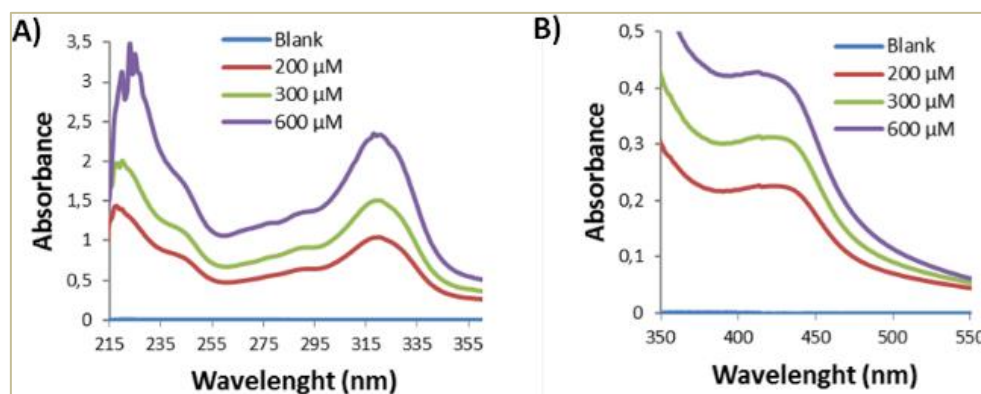


FIGURE 1. UV-visible spectra of purpurogallin derived from pyrogallol at different concentrations in a Tris-cacodylic acid buffer at pH 8.2.

is the xanthine oxidase-cytochrome c coupled assay [14]. Although widely employed, these spectrophotometric assays have some limitations for high throughput measurements using a plate reader, and the nitroblue tetrazolium (NBT) assay is limited by the relative insolubility of the formazan end-product [15]. The inefficiency of this method has contributed to the development of alternative methods to assay SOD activity based on other processes. Indeed, it is difficult to compare data obtained by using the different methods since even slight differences in the assay conditions render discrepant results.

A simple spectrophotometric assay was proposed by Marklund and Marklund [16]. This method is based on the ability of SOD to inhibit the autoxidation of pyrogallol at alkaline pH. Since the oxidation of pyrogallol leads to the production of a yellow-colored product called purpurogallin, the rate of change in the absorbance at 420 nm, which represents the rate of autoxidation of pyrogallol, can be conveniently followed by spectrophotometric techniques. As SOD activity inhibits pyrogallol autoxidation (by dismutation of superoxide), this process allows the quantification of the time course of SOD activity. Despite this method being simple and reliable, it is very sensitive to pH and temperature conditions, buffer composition, and even nature of samples, and is not suitable for reactions that follow a non-linear kinetic. In this SOD enzyme activity protocol, we describe a procedure to monitor pyrogallol autoxidation under different conditions, in order to optimize sensitivity, precision, reproducibility, linearity, iso-

form discrimination as well as to assess its inhibition kinetics, in a variety of biological samples.

2. METHOD PRINCIPLES

2.1. Assay Principle

The SOD activity assay was designed to quantitatively measure SOD activity in a variety of samples and experimental conditions. This assay allows measurements of all types of SOD activities, including Cu,ZnSOD, MnSOD, and ECSOD. This method is based on the ability of SOD to inhibit the autoxidation of pyrogallol at alkaline pH. Since the oxidation of pyrogallol leads to the production of a yellow-colored product called purpurogallin, the rate of increase of the absorbance at 420 nm, which can be conveniently followed by spectrophotometric techniques, is reduced by the presence of SOD activity, which removes superoxide and therefore inhibits the autoxidation of pyrogallol. This is reflected as a reduction in yellow product formation in SOD-containing samples.

2.2. Assay Improvement

Our method is based on the well-known spectrophotometric assay introduced by Marklund and Marklund, (1974) [16]. With the final purpose of improving linearity, sensitivity, precision, versatility, and reproducibility, we have assessed pyrogallol au-

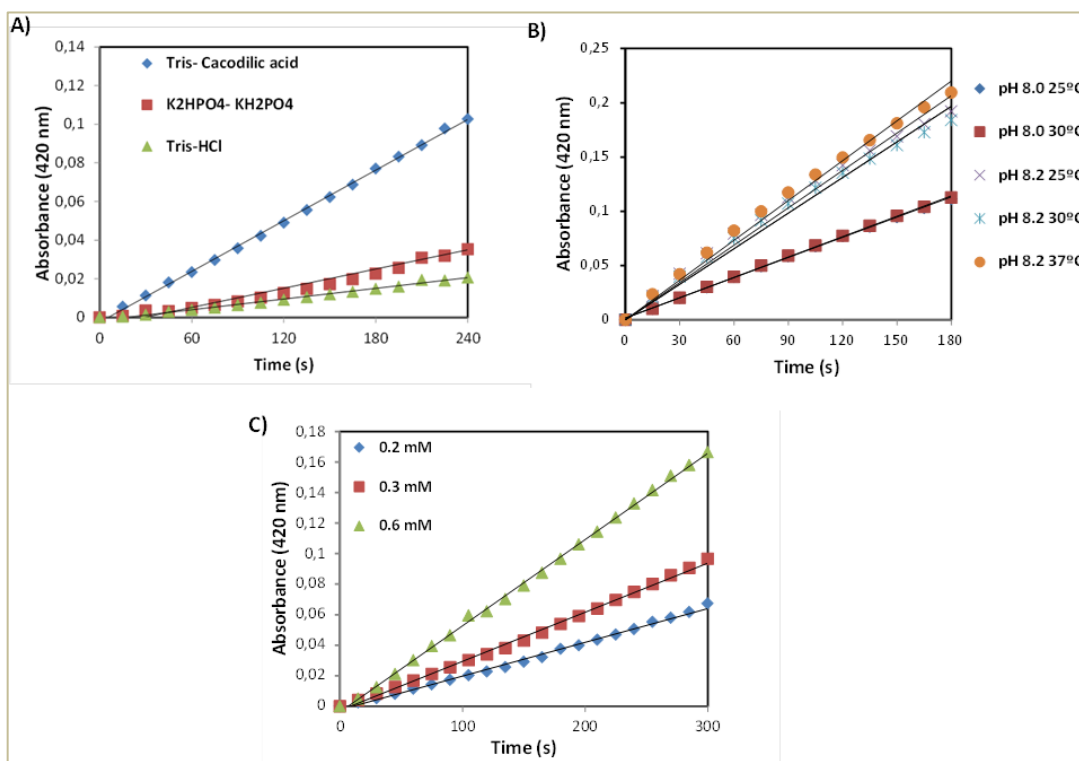


FIGURE 2. Effects of assay conditions on pyrogallol autoxidation. (A) Effect of buffer composition: representative experiment of pyrogallol autoxidation in different buffers at 37°C and 0.6 mM pyrogallol. (B) Temperature and pH effects: representative experiment of pyrogallol autoxidation at different temperatures (25, 30, and 37°C) and pH (8.0 and 8.2) in Tris-cacodylic acid buffer and 0.6 mM pyrogallol. (C) Effects of pyrogallol concentrations (0.2–0.6 mM): representative experiment of pyrogallol autoxidation in Tris-cacodylic acid buffer, pH 8.2, and 37°C.

toxidation under various conditions in order to optimize the method. Finally, we have also miniaturized the procedure to adapt the assay to multi-well plate readers, for the analyses of a large number of samples and conditions.

2.2.1. Monitoring Wavelength

Purpurogallin wavelength spectrum was obtained, following the autoxidation of pyrogallol, at pH 8.2 in Tris-cacodylic acid buffer with different concentrations of pyrogallol (Figure 1). The purpurogallin wavelength spectrum was characterized by the existence of two absorbance peaks at 220 and 320 nm in the ultraviolet (UV) range (Figure 1A) and a concentration-dependent stable plateau between 400 and 440 nm in the visible range (Figure 1B). For differ-

ent reasons (mainly unspecific UV absorption of cellular extracts), in our assay the autoxidation was essentially studied by measuring the absorbance at 420 nm, and the reaction rate is taken from the linear slopes in absorption/time plots seen for the first < 5 minutes after incubation with the sample.

2.2.2. Reaction Conditions: Buffers, Temperature and Pyrogallol Concentrations

Pyrogallol oxidation is strongly dependent on the pH of the reaction media, being inhibited at neutral to acid pH values. We first assessed the effects of reaction buffer composition on pyrogallol autoxidation kinetics using three alkaline buffers: Tris-cacodylic acid buffer (50 mM Tris, 1 mM DTPA), potassium phosphate buffer (100 mM K₂HPO₄ and 100 mM

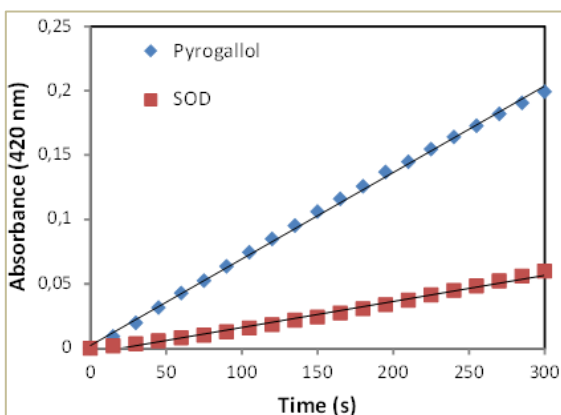


FIGURE 3. Representative example of inhibition of pyrogallol autoxidation by 2 units/ml bovine erythrocyte Cu,ZnSOD in 50 mM Tris-cacodylic acid buffer, containing 1 mM DTPA, pH 8.2, and 0.3 mM pyrogallol, at 37°C.

KH_2PO_4 , 1 mM DTPA), and Tris-chloride buffer (50 mM Tris, 1 mM DTPA), all at pH 8.0 and using 0.6 mM pyrogallol. The reaction was monitored at 420 nm every 15 s for 5 min at 37°C. Pyrogallol autoxidation rate was higher in the Tris-cacodylic acid buffer (**Figure 2A**). To confirm the effects of pH and temperature in the kinetic of pyrogallol autoxidation, we analyzed the effects of different pH and temperature in the Tris-cacodylic acid buffer, using a pyrogallol concentration of 0.6 mM. As shown in Figure 2B, the rate of autoxidation increased with pH and temperature. Pyrogallol autoxidation, both at pH 8.0 and 8.2, was higher at 37°C (**Figure 2B**), although at the more alkaline pH autoxidation ($[\text{pyrogallol}] = 0.6 \text{ mM}$) rate tends to lose the linear pattern after 3 min. Likewise, under the same temperature conditions (37°C), pyrogallol autoxidation was higher at pH 8.2. To confirm the suitability of the pyrogallol concentration in the reaction mixture, we next tested the autoxidation rate at different pyrogallol concentrations (0.2, 0.3, and 0.6 mM) in the Tris-cacodylic acid buffer, pH 8.2, and 37°C. The autoxidation rate increased linearly with pyrogallol concentration (**Figure 2C**).

Thus, we have observed that better sensitivity and reproducibility using the pyrogallol autoxidation method were obtained when experimental conditions were: 50 mM Tris-cacodylic acid 1 mM DTPA, 0.3

pyrogallol, pH 8.2, and incubated at 37°C. The reaction mixture was being monitored spectrophotometrically at 420 nm for 5 min after addition of pyrogallol.

2.2.3. SOD Assay

We finally checked for the ability of SOD to inhibit pyrogallol autoxidation using lyophilized bovine Cu,ZnSOD. At the optimal reaction conditions, the autoxidation of pyrogallol was inhibited by incorporation of purified bovine erythrocyte Cu,ZnSOD. In the example shown in **Figure 3**, pyrogallol autoxidation was inhibited about 74% by 2 units/ml of Cu,ZnSOD.

3. MATERIALS AND INSTRUMENTS

3.1. Materials

- (1) Cacodylic acid (Sigma-Aldrich, St. Louis, MO, USA)
- (2) Diethylenetriaminepentaacetic acid (DTPA) (Sigma-Aldrich)
- (3) Lyophilized bovine erythrocyte Cu,ZnSOD (Sigma-Aldrich)
- (4) Potassium phosphate-buffered saline (100 mM $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$), pH 8.0
- (5) Pyrogallol (Sigma-Aldrich)
- (6) Tris-chloride acid buffer (50 mM Tris adjusted to pH 8.0 with 0.5 N HCl)
- (7) Sodium cyanide (Sigma-Aldrich)

3.2. Instruments

- (1) UV/Vis spectrophotometer
- (2) Multi-well plate reader with absorbance filters around 420 nm

4. PROTOCOLS AND STEPS

4.1. Assay Description

Pyrogallol autoxidation assay is used to detect SOD activity. Briefly, pyrogallol (a final concentration of 0.3 mM) is added to cuvettes or wells containing 50 mM cacodylic acid, 1 mM DTPA, and the appropriate amount of sample. Reaction is initiated by addi-

tion of pyrogallol. After gently mixing (or shaking in multi-well plate readers) the reaction mixture is monitored spectrophotometrically at 420 nm every 15 s for 5 min. The decrease in the autoxidation rate of control (sample-free) is related to the activity of SOD present in the sample.

4.2. Preparation of Reagents

4.2.1. Reaction Buffer (50 mM Tris-Cacodylic Acid, 1 mM DTPA, pH 8.2)

First, to prepare a 10 ml stock solution of 50 mM DTPA, weigh 0.197 g DTPA, add 7 ml deionized water, dissolve (to facilitate dissolution add a few drops of 1 M Tris), and gauge to 10 ml. Store stock solution at 4°C. Then, to prepare 50 ml of 50 mM Tris-cacodylic acid, dissolve 0.345 g cacodylic acid in 25 ml deionized water and add 1 ml of the 50 mM DTPA stock prepared previously. Adjust pH to 8.2 with Tris and dilute to 50 ml with deionized water.

4.2.2. 30 mM Pyrogallol

To prepare 10 ml, weigh 0.0378 g of pyrogallol and dissolve in 0.5 N HCl. Store at 4°C under dark conditions. Discard after a maximum of 5 days.

4.2.3. 30 mM Sodium Cyanide

To prepare 10 ml, weigh 0.0147 g of sodium cyanide and dissolve in 10 ml deionized water.

Caution: Sodium cyanide is highly toxic since it is a powerful inhibitor of respiration, acting on mitochondrial cytochrome c oxidase and therefore causes blockage of the electron transport chain. Handle using a mask and gloves.

4.3. Biological Samples

For validation of the method, six types of samples from five different species (human, mouse, rat, turtle, and unicellular fungi) were used in the present study. All samples were obtained following the guidelines of the local ethics committee.

4.3.1. Mouse Brain Homogenates

Samples (0.1 g) of brain tissues (cerebellum, septum, and frontal cortex) were homogenized in 1 ml buffer

(50 mM Tris-HCl, pH 8.0), containing a cocktail of protease inhibitors (Roche, Barcelona, Spain) at 4°C. The tissue homogenate was then centrifuged at 500 g for 5 min at 4°C, and the resulting supernatant was collected and stored at –80°C until enzyme assays.

4.3.2. Sea Turtle Hematocrit and Plasma

Blood samples were obtained from loggerhead sea turtles (*Caretta caretta*). Samples were extracted from venous cervical sinus, and sodium heparin was added to the collector tube to prevent blood coagulation. Plasma and hematocrit fractions were isolated by centrifugation at 400 g for 5 min at room temperature and stored at –80°C until performing enzyme assays. Plasma samples were immediately aliquoted and frozen. Red blood cell extracts were prepared by homogenization in cold hypotonic buffer (20 mM Tris-HCl, pH 7.6) containing 1x protease inhibitors cocktail.

4.3.3. Cultured HT22 Cells

Mouse-derived HT22 cells were cultured in standard Dulbecco's modified Eagle's medium (DMEM), as described in Martín et al. [17], cells were collected after 30 h (app. 70% confluence) and process by homogenization in a cold hypotonic buffer (Tris-HCl 20 mM, pH = 7.6) containing 1x protease inhibitors cocktail. Homogenates were centrifuged at 900 g for 10 min, and the supernatants collected and stored at –80°C until assays.

4.3.4. Yeast Extracts

Yeast extracts were prepared from *Candida albicans*. Yeasts were grown at 37°C in a synthetic complete medium (0.67% yeast nitrogen base, 2% glucose) supplemented with amino acids as described by Burke et al. [18]. Solid media contained 2% agar. Yeast extracts were obtained by osmotically lysing the cells using a hypotonic lysis buffer (20 mM Tris-HCl, pH 7.6) containing 1x protease inhibitors cocktail. Samples were stored at –80°C until performing enzyme assays.

4.3.5. Human Cerebrospinal Fluid

Human cerebrospinal fluid (CSF) was obtained by lumbar puncture in the L3/L4 intersegment of the

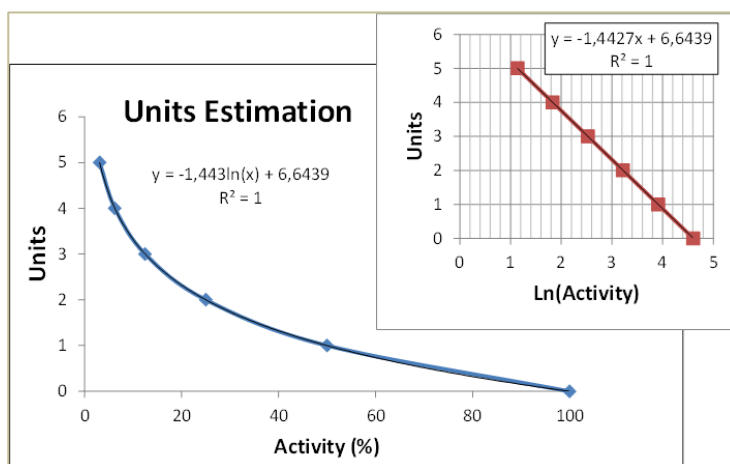


FIGURE 4. Estimation of SOD activity as a function of the percent activity compared to 100% control pyrogallol. In the insert is a representation plot for units vs ln(activity). Curve equations are indicated in each plot.

spinal cord. Afterwards, CSF samples were centrifuged at 1000 g for 10 min at 4°C and stored at –80°C in polypropylene tubes until enzyme assays.

4.3.6. Rat Platelets and Plasma

Whole blood was obtained by cardiac puncture using sodium heparin as an anticoagulant. Blood was centrifuged at 100 g for 30 min at room temperature to separate two phases: hematocrit and platelet-rich plasma (PRP). The supernatant formed by PRP was centrifuged again at 600 g for 15 min at room temperature to obtain another two phases: a supernatant corresponding to plasma and a pellet containing the platelets. Platelets were processed as described above for HT22 cells. Samples were stored at –80°C until enzyme assays.

4.4. Steps

4.4.1. Determination of Total SOD and MnSOD Activities in Spectrophotometer Cuvettes

- (1) Blank cuvette: Add 0.9 ml of reaction buffer and 0.1 ml of the specific buffer in which samples were diluted or water if samples were not diluted.
- (2) Control cuvette: Add 0.9 ml of the reaction buffer and 0.1 ml of the specific buffer. In the case of MnSOD assays, add 0.876 ml of the reaction

buffer, 0.024 ml of 30 mM NaCN, and 0.1 ml of the specific buffer in which the samples were diluted or water if samples were not diluted.

- (3) Samples cuvettes for determining total SOD activity: Add 0.9 ml of reaction buffer and 0.1 ml of sample. For determination of MnSOD activity, add 0.876 ml of reaction buffer, 0.024 ml of 30 mM NaCN, and 0.1 ml of the sample. Incubate with agitation for 10 min.
- (4) Add 10 µl of 30 mM pyrogallol in control and sample cuvettes. Do it slowly over the top of an angle of the cuvette. Mix the content of cuvettes and proceed to measure the absorbance.
- (5) Measure the absorbance at 420 nm in an adequate spectrophotometer every 15 s for 3–5 min at 37°C (mammalian samples) or room temperature (other samples).

4.4.2. Determination of Total SOD Activity in Multi-Well Plate Readers

- (1) Blank well: Add 0.18 ml of the reaction buffer and 20 µl of the homogenization buffer or water if samples were not diluted.
- (2) Control well: Add 176 µl of the reaction buffer 20 µl of the homogenization buffer or water if samples were not diluted, and 4 µl of 30 mM pyrogallol. For MnSOD assays, add 171 µl of the reaction buffer and 5 µl of 30 mM NaCN, and 20

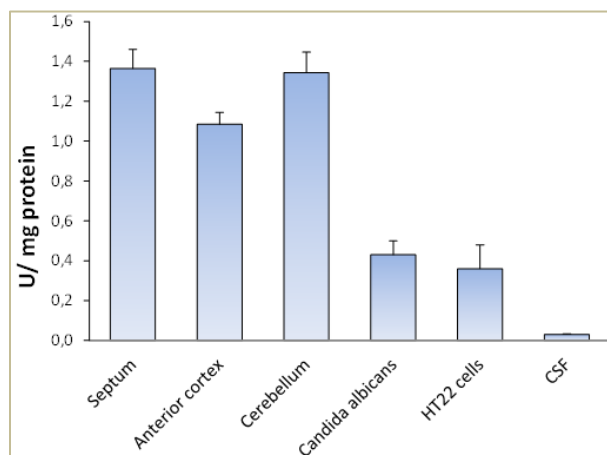


FIGURE 5. SOD activity in different mouse brain regions, yeast extracts, HT22 cells, and human cerebrospinal fluid (CSF). Activities are expressed in units/mg protein as mean \pm SEM from at least 3 separate experiments.

μ l of the specific buffer in which samples were diluted or water if samples were not diluted.

- (3) Sample wells: Add 176 μ l of the reaction buffer and 20 μ l of the sample. For MnSOD assays, add 171 μ l of the reaction buffer, 5 μ l of 30 mM NaCN, and 20 μ l of the sample. Incubate with agitation for 15 min.
- (4) Set plate reader temperature to 37°C or room temperature depending on the sample source (see above). Add 4 μ l of 30 mM pyrogallol to all wells to begin the assay. Place the multi-well plate in holder and shake for 1 min. Measure absorbance at 420 nm in a multi-well plate reader every 15–20 s for 5 min at 37°C or room temperature.

5. CALCULATION

- (1) Determine the rate of increase in absorbance/min for the control and for the test samples as the slope (S_c) of the control from the absorbance-time plot by simple lineal regression. Repeat the procedure for each plot sample in the experiment (S_{sIn}). Be careful to choose lineal ranges and avoid potential outliers. It is not necessary to take the whole range of measurements, but those

that are clearly lineal ($R^2 > 0.95$). These slopes give a measurement of absorbance changes per min.

- (2) Determine the percent of activity for the test samples. Considering S_c as 100% autoxidation, the inhibition by the presence of SOD in the test samples are calculated as: Activity (%) = $(S_{si} \div S_c) \times 100$; Inhibition (%) = $100 - \text{Activity} (\%)$.
- (3) Calculation of SOD Activity: One unit of SOD inhibits the rate of increase in absorbance at 420 nm by 50% under current assay conditions. Thus, as the change in activity (and inhibition) is not lineal but exponential, the number of units is calculated by application of expressions shown in **Figure 4**. Units = $-1.4427 \times \ln(\text{Activity}) + 6.6439$.

As an example, in the plot illustrated in **Figure 3** for lyophilized SOD, the activity was 26% of control pyrogallol (74% inhibition), and by substitution in equations from **Figure 4**, a value of 1.97 units was obtained, which was very close to the nominal amount of SOD lyophilized (2 units) included in the assay. It is strongly recommended to dilute samples when the percent of inhibition is higher than 80%.

6. MONITORING PYROGALLOL AUTOXIDATION IN BIOLOGICAL SAMPLES

We have validated the method described above using different biological samples. Results shown in **Figure 5** illustrate an experiment where pyrogallol autoxidation was modified by incorporation of natural sources of SOD using reaction conditions: Tris- cacodylic acid buffer, pH 8.2, at 37°C and 0.3 mM pyrogallol. Biological samples included different mouse brain areas, human cerebrospinal fluid, cultured HT22 cells, and yeast extract. At first glance, it can be seen that the SOD activity is greater in brain regions and lowest in the cerebrospinal fluid.

We have used rat PRP to illustrate the different SOD activities (**Figure 6**). Thus, in PRP preparations, a total SOD activity value of 0.67 ± 0.02 units was obtained. When the different isoenzymes were studied separately, a value of 0.35 ± 0.04 units for Cu,ZnSOD and 0.13 ± 0.01 units for MnSOD were obtained. In rat plasma, a value of 0.11 ± 0.01 units corresponding to ECSOD.

Certain biological samples may contain molecules with significant absorbance at 420 nm, which will

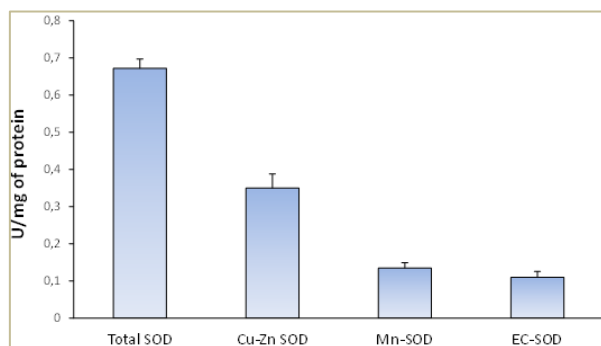


FIGURE 6. Activities of SOD isozymes in PRP preparations. Activities are expressed in units/mg of protein as mean \pm SEM from at least 3 separate experiments.

interfere with the determination of pyrogallol autoxidation and consequently the measurement of SOD activity. Such is the case of red blood cell samples, in which the presence of hemoglobin induces a contamination on the purpurogallin signal, because hemoglobin has a significant light absorption at this wavelength, which may saturate the spectrophotometric signal. Although the optimal conditions of our test were to use 420 nm, in these cases, the measuring wavelength can be shifted towards lower or higher lambda values in the plateau phase of purpurogallin spectrum (Figure 7). In the case of red blood cells, this could be circumvented by shifting the measuring wavelength to 400 nm.

7. DISCUSSION OF ADVANTAGES AND LIMITATIONS

Our assay focuses on the method developed by Marklund and Marklund, based on the inhibition of pyrogallol autoxidation caused by the presence SOD. Pyrogallol (benzene-1,2,3-triol) reacts with dioxygen (O_2) in weakly alkaline solutions to form purpurogallin (2,3,4,6-tetrahydroxy-5*H*-benzocycloheptene-5-one) [19] and generates superoxide, which in turn favors pyrogallol autoxidation. In the presence of SOD, which catalyzes the dismutation of superoxide to form O_2 and hydrogen peroxide, the formation rate of purpurogallin decreases in an SOD concentration-dependent manner.

To establish an appropriate reaction medium favoring pyrogallol autoxidation, we assayed three different buffers described previously in the literature to determine SOD activity, namely, potassium phosphate buffer [20], Tris-chloride buffer [21], and Tris-cacodylic acid buffer [16]. Our data show that pyrogallol autoxidation is greater, reproducible, and showing linearity in the Tris-cacodylic acid buffer at slightly alkaline pH. Indeed, using different techniques SOD activity has been shown to be very sensitive to pH [22, 23], being reversibly decreased at weak alkaline, neutral, or acidic pH, and irreversibly inhibited at strong alkaline pH due to the alkali-driven denaturation of the SOD protein. In this work, we find a pH window in the range of pH 8.0 to 8.2 to obtain optimal SOD activity. Pyrogallol autoxidation kinetics also depends on the pyrogallol concentration, losing linearity at higher concentrations. Thus, the key to obtain satisfactory and reproducible results lies in the precise control of reaction media parameters [24]. Our experimental conditions increase the resolution of results compared to the original Marklund and Marklund method. In terms of chemical kinetics, our data demonstrate very good linear relationships between the value of purpurogallin absorbance at 420 nm and the reaction time for pyrogallol at 0.3 mM. These conditions allow us to compare SOD activities in unrelated biological samples in the same assay, and also to observe the process directly. Other techniques make use of UV wavelengths to monitor the assay [20], as purpurogallin has a maximum absorbance peak at 320 nm (see Figure 1A). However, as we have shown, this approach becomes inaccurate as many cellular components existing in the biological extracts exhibit significant light absorption at wavelengths in the UV range. Thus, although the quantum yield of purpurogallin is lower at 420 nm than at 320 nm, the election of wavelengths in the visible range (i.e., 420 nm or 400 nm) decreases contribution of spurious interferences in biological samples.

An important issue concerning SOD analyses is the discrimination between the cytosolic and mitochondrial isoforms, i.e., Cu,ZnSOD and MnSOD. Millimolar concentrations of cyanide (1–2 mM) have been reported to irreversibly inhibit Cu,ZnSOD by 97–99% at alkaline pH [25]. In contrast, MnSOD is not subject to inhibition by cyanide. Thus, by introducing simple alterations in the reaction media, based on their differing sensitivity towards cyanide

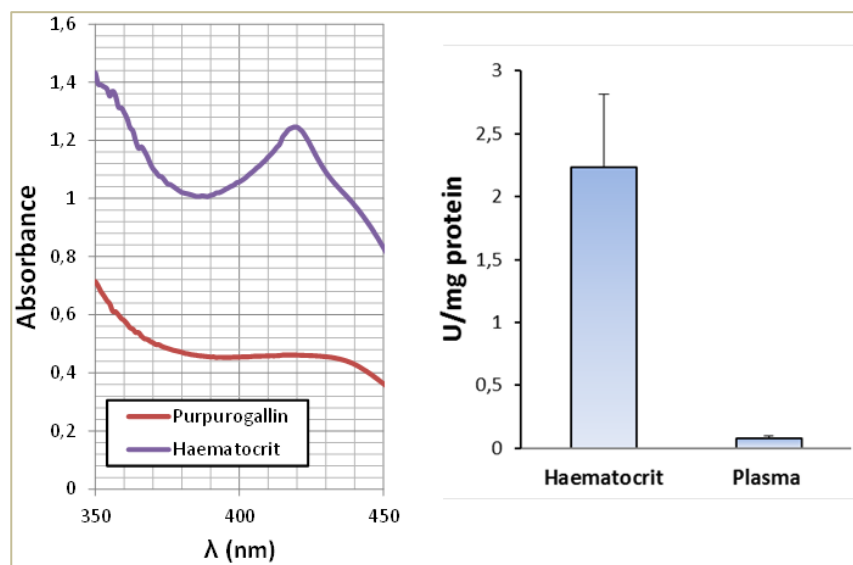


FIGURE 7. SOD activities in sea turtle hematocrit and plasma. Left: comparative absorbance spectra of hemoglobin and pyrogallol. Right: SOD activities as measured at 400 nm. Activities are expressed in units/mg of protein as mean \pm SEM from 17 different individuals.

inhibition [7], we could determine different SOD isozyme activities in the same samples. This augments the versatility of the procedures described in this article.

8. CONCLUSION

In conclusion, the modified pyrogallol-based assay described in the present paper meets the needs for sensitive and versatile analyses of SOD activities, applicable to the analysis of total and isoenzyme-specific SOD. The method has been validated for different biological samples of great interest in the fields of ROS biochemistry, plant and animal physiology, and clinical chemistry, among others. Also, this improved method has higher linearity, sensitivity, precision, and reproducibility than the original method by Marklund and Marklung and the NBT method. The improved protocol requires only standard laboratory equipment and inexpensive chemical reagents. It is therefore considered to be a cheap, sensitive, accurate, and versatile method suitable for the quantification of the superoxide-scavenging activities of SOD isozymes in most types of biological samples.

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