A computer program to calculate superoxide dismutase activity in crude extracts

Ricardo Vázquez-Juárez\textsuperscript{a,b}, Francisco Vargas-Albores\textsuperscript{a} and J.L. Ochoa\textsuperscript{a}

\textsuperscript{a}Centro de Investigaciones Biológicas de B.C.S., La Paz, México, and \textsuperscript{b}Department of General and Marine Microbiology, University of Göteborg, Göteborg, Sweden

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Summary

By virtue of the instability of $O_2^-$, most of the methods used for measuring superoxide dismutase (SOD) activity are indirect and produce a non-linear response with respect to the amount of SOD enzyme. For this reason a linearization procedure must be used to estimate enzyme activity. A computer method written in Turbo Pascal for IBM-PC or compatibles is presented which simplified calculations. The program applied to methods using different $O_2^-$ generation systems (i.e. fluorescent light, xantine–xantine oxidase, etc.) or indicating scavengers (cytochrome c or Nitro Blue Tetrazolium). It could also be used for both crude extracts or samples at different steps of purification.

Key words: Superoxide dismutase; Enzyme activity; Computer program

Superoxide dismutases (SOD) is a group of enzymes in charge of the elimination of toxic oxygen (superoxide radicals) produced as a byproduct in respiring organisms [1]. In recent years, different methods have appeared describing several alternatives to measure SOD activity (summarized in [2,3]). The most common procedure is based on the inhibition of a free radical superoxide ($O_2^-$) induced reaction [4]. This combines a ($O_2^-$) generating system (xantine-xantine oxidase, fluorescent light, etc.) with an indicating scavenger (IS), such as cytochrome or Nitro Blue Tetrazolium (NBT) whose reduced forms are coloured, and finally the inhibiting enzyme SOD. The SOD enzyme intercepts the flux of $O_2^-$, therefore avoiding the reduction of the indicating scavenger. In this context, one enzyme unit is defined as the amount of SOD enzyme that causes a 50% inhibition in the redox reaction [2]. Since it is assumed that a non-purified enzyme may contain other components which provide an oxygen-independent route of electron transfer from the generating system to the indicating scavenger, 100% of the inhibition is never reached when crude prepara-
Fig. 1. (a) Inhibition of the reduction of NBT by SOD. (b) Linearization of SOD inhibition of NBT reduction.

Inhibitions of the enzyme are used (Fig. 1a). Hence, the percentage of IS reduction is not in direct proportion to the amount of SOD enzyme, and the determination of the 50% inhibition might be troublesome.

An easier estimation of SOD activity can be made according to Assada [3], taking into consideration that the superoxide radical can react in three ways: (a) by donation of an electron to the indicating scavenger (Cyt. C or NBT)

\[ v = \frac{d[\text{IS Red}]}{dt} = K_c [\text{IS Ox}][O_2^-] \]  

(1)

(b) by spontaneous disproportionation not catalyzed by superoxide dismutase

\[ d[O_2^-]/dt = K_s [O_2^-] \]  

(2)

(c) by disproportionation catalyzed by superoxide dismutase

\[ d[O_2^-]/dt = k_e [\text{SOD}][O_2^-] \]  

(3)

Under the common experimental conditions employed for determining SOD activity,
the spontaneous disproportionation rate of the radical is negligible; therefore, \((O_2^-)\) disproportionation is virtually produced at a rate given by:

\[ V = -d[(O_2^-)]/dt \]

or \( V = k_e[\text{IS. Ox.}][O_2^-] + k_e[\text{SOD}][O_2^-] \) \(\text{(4)}\)

By dividing equation (4) by equation (1), we obtain:

\[ \frac{V}{v} = 1 + \frac{K_e[\text{SOD}]}{K_e[\text{IS Ox}]} \] \(\text{(5)}\)

where \(v\) and \(V\) are the IS reduction rate in the absence and presence of enzyme; [IS Red] and [IS Ox] indicate the concentration of reduced and oxidised IS respectively; and \(K_e, K_s, \text{ and } K_c\) are rate constants.

Since the initial rate of indicator scavenger reduction is a function of \([O_2^-]\) (first order reaction), then, \(K_e/K_c\) [IS Ox] can be considered as a global constant \( (K')\) and therefore:

\[ V/v = 1 + K' [\text{SOD}] \] \(\text{(6)}\)

Equation (6) indicates that the ratio of IS reduction rate in the presence and absence of the enzyme \((V/v)\) is proportional to the concentration of the enzyme. Since the experimental data offered by the test actually indicates the amount of reduced IS, it is necessary to calculate the reciprocal values in order to elaborate a linear plot for determining the amount of enzyme capable of producing the 50% inhibition of IS reduction, which corresponds to one enzyme unit \([2]\). To illustrate this, we present data related to the estimation of the SOD activity in a yeast strain presumably belonging to Debaryomyces Hansenii designated, as C-11, isolated from the Pacific coast of Southern State of Baja California, México \([6]\). The procedure for extraction was as follows: 4 g of lyophilized yeast were treated with 12 ml of chloroform-methanol (1:2 \(v/v\), under continuous stirring, at 10°C; after 3 h, the sample was centrifuged at 3500 rpm for 10 min and the supernatant collected. The sediment was then extracted with 50 mM phosphate buffer pH 7.8, for 3 h with the aid of a magnetic stirrer and centrifuged again as above. The protein content of the supernatant was found by Lowry’s method \([5]\). Enzyme activity was determined by mixing different aliquots of the supernatant with 2 ml of 50 mM phosphate buffer, pH 7.8, containing \(1.17 \times 10^{-6}\) M of riboflavin, 0.01 M of methionine, \(2 \times 10^{-5}\) NBT and 0.1 M EDTA, the enzyme activity was determined when the control tube (without enzyme) reached a value of 0.2 absorbance units at 560 nm in a Spectronic 2000 spectrophotometer. The inhibition of NBT reduction, or decrease in absorbance units, was calculated according to the following equation:

\[ \text{Inhibition } \% = \frac{(\text{OD } V - \text{OD } v)/(\text{OD } V)}{100} \]
An accurate estimation of the enzyme specific activity, according to McCord and Fridovich [1], means first, the determination of the range in which inhibition percent is a function of extract volume; and second, a correction of the values thus obtained, assuming that the maximum value corresponds to 100% inhibition. Using the corrected values within such a range, and plotting the ratio \( V/v \) versus the amount of extract volume used, we finally obtain a linear function. The volume equivalent to one unit (\( V_u \)), may be obtained (in \( \mu l \)) by interpolation when \( V/v \) is equal to 2, and thus, the specific activity SA calculated by:

\[
SA = \frac{1000/(mg\ protein/ml)}{(V_u)}
\]

One different way to calculate the Units in crude extracts has been suggested by Ysebaert [7]. He used the same model as equation 6 but expressed [SOD] as (SOD units/ml) (1/dilution in cuvette). Then by plotting \( V/v \) \( V/s \) (1/dilution in cuvette), the slope represents \( k \) (SOD units/ml). Considering that one activity unit corresponds to the amount of enzyme contained in an assay volume that reduces \( v \) to one half of \( V \) \( (v = V/2) \), then \( k \) equals assay volume and then activity units per milliliter = slope/assay volume. By using this procedure we have obtained similar results.

The above calculations can be simplified by means of a computer program designed for the determination of SOD activity. The program is written in Turbo Pascal 5.0, for IBM or compatibles, using a DOS version 3.0 or later. On inserting the diskette, the program is called by typing SOD. Up to ten different dilutions with their corresponding OD values can be entered at random using arrow keys. <Enter> is used to store the values, and <Esc> to abort the input, restoring the previous value. An important simplification of the SOD program is that only three columns are needed: The first is to type the enzyme volume, whilst the other two take the corresponding OD values. The number of dilutions, or data pairs, must be assigned.

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### Table

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<th>Volume</th>
<th>Abs 1</th>
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<th>Mean</th>
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<th>% Inh C</th>
<th>V/v</th>
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<td>8.364</td>
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</table>

Name of Experiment : Batch 1
Protein conc. : 1.24 mg/ml
Dilution Factor : 2.00
Number of Data : 7

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Fig. 2. Using the <F2> key to calculate the mean of absorbance (Mean), the percent of inhibition (% inhib), the percent of corrected inhibition (% inh C) and the \( V/v \) ratio (\( V/v \)).
by means of <F1> before a calculation key is used. With this function one can also edit or modify the name of the experiment, protein concentration and dilution factor. The <F2> key performs the mathematical operations needed to establish the mean of absorbance, the % inhibition, the % corrected inhibition and the $V/V$ ratio (Fig. 2). Using the <F3> key, the program makes the necessary calculations to determine the correlation coefficient, intersection point, slope and volume necessary for obtaining 50% inhibition, all of which appear in a second screen (Fig. 3). The program evaluates these results, selecting from the original data the range that provides the best correlation coefficient which appears highlighted on the screen. In addition, the specific activity is calculated and displayed.

Using the <F4> key, the tables containing all data and calculations made by the program, which appear in Figs. 2 and 3, may be directly printed. The best correlation coefficient is marked with an asterisk. No previous calculation (<F2> and <F3>) are necessary to print the results. The <F5> key permits one to clear the table for entering other data. Finally, the <F6> key ends the program and returns it to DOS.

Fig. 1 shows a plot of % inhibition versus extract volume (using our example data), and linearization of the function according to Assada [3]. As can be easily inferred, the program allows us to calculate the SOD enzyme activity in crude or purified preparations in a rapid and efficient manner. We hope that students as well as interested researchers will find this approach useful. All of those interested in receiving a copy of the program may do so by sending a diskette and a letter promising not to duplicate the program and making reference to this note in any application.

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References