Caution on the use of oxygen electrode-based assay for superoxide dismutase activity in crude preparations

Superoxide dismutases (EC 1.15.1.1) are a family of metalloenzymes that catalyse dismutation of superoxide radicals into oxygen and hydrogen peroxide. Growing literature in this area has signified the importance of superoxide dismutase (SOD) in imparting oxidative stress tolerance to plant and animal systems[1,2]. This necessitated the development of a number of assay procedures to meet the experimental requirements[3].

Flohé and Ötting[3] and Beyer and Fridovich[4] critically reviewed a number of procedures to assay SOD and highlighted some of the associated problems. Working with crude extracts of plant leaves poses a problem of green colour in the reaction medium, which interferes while assaying with any procedure based on spectrophotometry, e.g. NBT (nitro blue tetrazolium) reduction assay. Assay based on oxygen electrode[5] eliminated some of the problems linked with other assays[4] and the problem of colour in crude preparations was accounted for. In this assay procedure, riboflavin was photoreduced in the presence of EDTA (ethylene diamine tetra acetic acid, di-sodium salt); reoxidation of reduced flavin generated superoxide radicals utilizing oxygen molecules from the reaction medium. An oxygen electrode was used to monitor this depleted oxygen. In the presence of SOD superoxide radicals are disproportionated to oxygen, leading to the replenishment of the oxygen concentration of the medium. Therefore, an 'apparent inhibition' of oxygen uptake is recorded by the oxygraph. The degree of inhibition in oxygen uptake was used to calculate SOD activity.

However, the procedure suffers from a major drawback, particularly when applied to crude preparations. As mentioned earlier in a SOD catalysed reaction hydrogen peroxide is also generated. This hydrogen peroxide is likely to be decomposed into oxygen and water by the action of catalases (EC 1.11.1.6) present in the crude extract. Thus depleted oxygen from the reaction medium would be replenished by SOD as well as catalases activity. Consequently, oxygraph will record more inhibition in terms of oxygen uptake, leading to overestimation of SOD activity. Moreover, the procedure[5] was reported without comparing it with any well-established procedure.

Our effort in the present communication is to study the interference caused by catalases in SOD estimation by oxygen electrode and NBT reduction procedures. Initially, oxygraph-based procedure was tested for its reliability by comparing with NBT reduction procedure, which is a very well accepted method for SOD assay.

Crude extract from leaves (1 g leaf homogenized in 10 ml of 50 mM potassium phosphate buffer, pH 7.8 containing 400 mg PVPP (polyvinylpolypyrrolidone); supernatant was used after centrifugation at 15,000 rpm for 20 min at 4°C) of tea (*Camellia sinensis*), pea (*Pisum sativum*) and barley (*Hordeum vulgare*) and purified SOD from tea leaves was used to assay by NBT reduction and oxygraph procedures[4,5]. SOD from crude extract of tea leaves was purified by salting out at 30–60% ammonium sulphate saturation. Precipitate was dissolved in phosphate buffer (pH 7.8, 10 mM), dialysed for 24 h against the same buffer and fractionated on a DEAE (diethylaminoethyl) cellulose column using 50, 200 and 500 mM KCl. The fraction with the highest specific activity for SOD (purified SOD), as determined by NBT reduction procedure, was used for our purpose. The purified enzyme was size-fractionated through a TSK column on HPLC (Data System 450, Kontron Instruments, Switzerland) using phosphate buffer (pH 7.8, 10 mM) at a flow rate of 3.0 ml/min and a major peak with highest SOD activity (extra purified enzyme) was used for experiments involving addition of external catalase (Sigma Chemicals; cat# C 2001).

Purified SOD was assayed along a temperature range of 15–35°C. Both the procedures showed maximum activity for SOD at 30°C with a very high correlation ($r = 0.824$, data not shown). The two procedures responded similarly to different SOD concentrations ($r = 0.982$, data not shown) along a large range of protein concentration. There was a shift in pH optima for SOD by the two procedures. NBT reduction method displayed maximum SOD activity at pH 7.8 whereas, oxygen electrode procedure revealed the best results at pH 7.4 (data not shown).

Table 1. Determination of SOD activity in crude extract and in purified enzyme by NBT reduction and oxygen electrode procedures[a]

Enzyme preparation	SOD activity as per cent inhibition					
	NBT reduction procedure[b]			Oxygen electrode procedure[c]		
	A	B	C	A	B	C
Tea[d]	20.28 ± 4.80	19.48 ± 4.20	21.00 ± 3.65	48.35 ± 0.48	47.96 ± 0.40	61.56 ± 0.18
Pea[d]	22.24 ± 1.90	23.75 ± 1.60	22.86 ± 1.95	70.18 ± 0.87	71.62 ± 1.11	88.64 ± 1.21
Barley[d]	43.21 ± 4.90	44.54 ± 2.30	43.93 ± 5.00	73.00 ± 0.35	72.41 ± 0.53	90.35 ± 0.49
Purified enzyme	46.29 ± 0.66	45.95 ± 0.51	46.09 ± 0.19	29.50 ± 0.15	45.38 ± 1.60	70.77 ± 0.20

[a] Reactions were carried out at 30°C; incident radiation, 1000 $\mu E\ m^{-2}\ s^{-1}$; separate blanks (with boiled enzyme) were prepared for each enzyme preparation.
[b] Reaction medium: 0.05 M potassium phosphate buffer (pH 7.8) containing 5.7×10^{-5} M NBT, 9.9×10^{-3} M methionine, 1.17×10^{-6} M riboflavin and 2.5×10^{-2}% (w/v) Triton X-100 in a total volume of 3 ml.
[c] Reaction medium: 5×10^{-5} M riboflavin, 1.5×10^{-4} EDTA and 0.5 µM KCl in a total volume of 3 ml of 0.05 M potassium phosphate buffer (pH 7.4).
[d] Leaf material was used to prepare crude extract.
A = no catalases or hydrogen peroxide was included during assay; B = catalase (3 U; Sigma Chemicals; cat# C 2001) was included in the reaction medium; C = hydrogen peroxide (75 nmole) and catalase (3 U; Sigma Chemicals; cat# C 2001) added in the reaction medium. ± represents S.E. values for three separate determinations.

The effect of sodium cyanide on inhibiting SOD activity was well recorded by the two procedures.

Although the correlation between the two procedures was very high, NBT reduction procedure always recorded more inhibition than oxygen electrode-based procedure when purified enzyme was used (Table 1). Such observed variations in enzyme activity by the two different procedures are not unusual. In fact, Beyer and Fridovich[4] reviewed a large number of procedures for SOD assay and realized a vast difference in the amount of SOD required for 50% inhibition (0.004–0.73 μg SOD/ml) and pH optima (0.2–10.2) for various assay systems. Our results have shown that the amount of protein required for 50% inhibition by oxygen electrode procedure is about 1.5 times higher than that required for NBT reduction test.

While performing assay with catalases free, extra purified SOD enzyme, it was interesting to note that the addition of 3 U catalase (Sigma Chemicals; cat# C 2001) in the reaction medium showed a further inhibition in the rate of oxygen uptake by oxygraph procedure, but had no effect on NBT reduction (Table 1). Thus, the added catalase catalysed the decomposition of hydrogen peroxide into oxygen and water and the oxygraph recorded this change. Since NBT reacts directly with the superoxide radical and the oxygen molecules *per se* have no effect on it, hence no change was detectable by the NBT reduction method. Hydrogen peroxide in the reaction medium will be generated not only by the action of SOD, but the possibility also exists that self-dismutation of superoxide radicals would generate hydrogen peroxide[6], leading to further release of oxygen by the action of catalases.

When SOD was assayed in crude extracts of tea, pea and barley by the two procedures, inhibition recorded was lower by 58.0, 68.31 and 40.8%, respectively in the NBT reduction method compared to oxygraph-based assay procedures. Our estimations showed very high catalases activity in the crude extracts. This probably explains a higher 'apparent inhibition' recorded by the oxygraph, as discussed earlier. Addition of external catalase in crude extracts had no effect on SOD estimations by the two procedures. Probably, catalases activity in the crude extracts was already present in optimum quantity.

In a separate experiment (Table 1), SOD activity by the two procedures was assayed in crude extract as well as in extra purified enzyme in the presence of externally added hydrogen peroxide (75 nmole) and purified catalase (3 U; Sigma Chemicals; cat# C 2001). The NBT reduction procedure did not show any difference in SOD activity, whereas oxygraph procedure showed further inhibition that was attributed to the externally added hydrogen peroxide. Inhibition thus produced was very much pronounced in case of purified enzyme (139.8% more inhibition) compared to that observed with the crude extract (23–27% more inhibition). While it justifies our hypothesis further, the results with crude extract point out that apart from catalases other hydrogen peroxide scavenging systems, e.g. peroxidases, which would not release any oxygen molecule during catalysis, might also play a role. However, their contribution will depend on the availability of the substrate(s) in the crude extract. Catalatic or peroxidatic activity largely depends upon leaf age, environmental factors and so on. Hence, results obtained with the crude preparations using oxygraph may be misleading.

Some of the known inhibitors of catalases like salicylic acid[7] or aminotriazoles[8] inhibit catalases only partially, while cyanide inhibits catalases as well as copper and zinc containing superoxide dismutase[9]. It was not possible to inhibit either of the enzymes completely to show the role of catalases in overestimation of SOD activity. Therefore, additive rather than the subtractive approach was followed to solve the problem.

While the oxygen electrode-based assay procedure appears to be simple, it does not involve any expensive chemical like NBT and is ideal for coloured preparations, the presence of catalases and other interfering agents, e.g. peroxidases makes it impractical to work with crude preparations. However, the method could be used with highly purified SOD preparations.

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SANJAY KUMAR
PRADIP KUMAR KUNDU
PARAMVIR SINGH AHUJA

Biotechnology Division,
CSIR Complex Palampur,
P.B. No. 6,
Palampur 176 061, India

D. Bhatnagar's response:

The determination of superoxide dismutase (SOD) by oxygen electrode (*Curr. Sci.*, 1995, **68**, 960–962) has not been presented for crude preparation and can be used only with purified enzyme. The crude homogenates cannot be used in this method due to high amounts of peroxidase and catalase and other proteins which should be removed. However, when purified enzyme was used, addition of low concentration of cyanide (0.5 μM) to the reaction mixture inhibit catalase completely without affecting measurement of SOD activity. Low concentration of cyanide (5–50 μM) has been used to inhibit peroxidase and cytochrome oxidase in crude samples while high concentration of cyanide (1–2 mM) has been employed in the differential cyanide inhibition assay to inhibit CuZn SOD and permit the quantitation of both CuZn SOD and Mn SOD (Iqbal, J. and Whitney, P., *Free Rad. Biol. Med.*, 1991, **10**, 69–77). In crude preparations due to the presence of catalase, decomposition of hydrogen peroxide into oxygen will lead to erroneous determination of SOD.

D. BHATNAGAR

School of Biochemistry,
Devi Ahilya Vishwavidyalaya,
Takshila Campus, Khandwa Road,
Indore 452 001, India