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A modified spectrophotometric assay of superoxide dismutase using nitrite formation by superoxide radicals

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A simple inexpensive colorimetric assay system for SOD is reported. The method involves generation of superoxide radical by photoreduction of riboflavin and its detection by nitrite formation from hydroxylamine hydrochloride at 543 nm. The method is more sensitive than the NBT method and less sensitive than the NADH method.

Superoxide dismutase (SOD, EC 1. 15. 1.1) protects cells by dismutating superoxide radicals to hydrogen peroxide and molecular oxygen. Almost all assays for SOD are based on indirect methods which involve scavenging of superoxide radicals by SOD. In general, SOD assay systems have two components: one is superoxide radical generating system and the second one is superoxide radical detecting system. Enzymatic (xanthine:xanthine oxidase), chemical ($MnCl_2$ and EDTA) and photochemical (riboflavin) methods are used to generate superoxide radicals[1,2] while luminometric, polarographic and chemical techniques are used to detect superoxide radicals[3,4]. In earlier studies where nitrite formation was used as a detection system for superoxide radicals, xanthine-xanthine oxidase was used as superoxide radical generating system[5]. In the present study superoxide radical generation by photoreduction of riboflavin[3] is combined with nitrite formation from hydroxylamine hydrochloride to detect superoxide radicals[5]. In brief, superoxide radicals are allowed to react with hydroxylamine hydrochloride to produce nitrite. The nitrite in turn reacts with sulphanilic acid to produce a diazonium compound which subsequently reacts with napthylamine to produce a red azo compound whose absorbance is measured at 543 nm (Fig. 1). Superoxide dismutase scavenges superoxide radicals produced by photoreduction of riboflavin. Therefore, nitrite formation in the reaction is inversely proportional to the amount of SOD. For comparative purposes, the enzyme activity was also assayed according to NBT[3] and NADH[4] methods.

The standard graph for nitrite detection by Greiss reagent was linear in the concentration range of 5 to 50 μM of sodium nitrite. The optimum substrate concentration and *p*H for maximum amount of $O_2^{\bullet-}$ generation was determined (Table 1). Effect of different durations of light exposure on generation of superoxide radical by photo-reduction of riboflavin

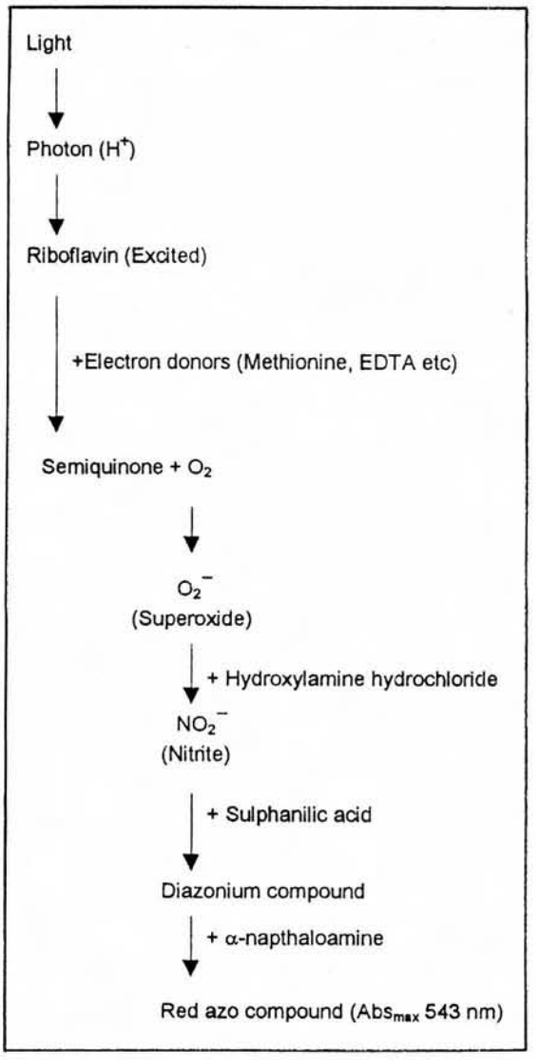

Fig. 1—Schematic representation of the nitrite method for determination of SOD activity

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Table 1—Composition of SOD assay system

Components	Stock concentration	Volume (ml)
Phosphate buffer *p*H 7.4	50 m*M*	1.110
L-Methionine	20 m*M*	0.075
Triton X-100	1% (v/v)	0.040
Hydroxylamine hydrochloride	10 m*M*	0.075
EDTA	50 μ*M*	0.100
Sample	30-40 μg protein	0.100
Riboflavin	50 μ*M*	0.080

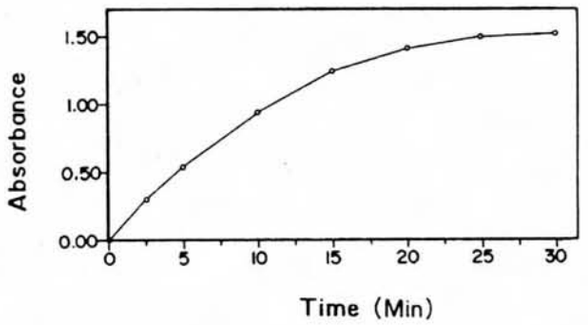

Fig. 2—Time dependent increase in generation of superoxide by photoreduction of riboflavin detected as nitrite formed by reaction with hydroxylamine [Data are expressed as mean ± S.D. of 6 observations]

determined by nitrite formation from hydroxylamine is depicted in Fig. 2.

On the basis of the above results in Table 1 the composition of the reaction mixture for measuring SOD activity by nitrite method is proposed. In this method 1.4 ml aliquots of the reaction mixture were taken in test tubes. The test sample (100 μl) was added to all tubes followed by a brief pre-incubation at 37°C for 5 min. Next, 80 μl of riboflavin was added to all tubes. The tubes were exposed for 10 min to two 20 W-Philips fluorescent lamps fitted parallel to each other in an aluminium foil coated wooden-box. The control tube contained equal amount of buffer instead of sample. The sample and its respective control were run together. At the end of the exposure time, 1 ml of Greiss reagent was added to each tube and the absorbance of the colour formed was measured at 543 nm. One unit of enzyme activity is defined as the amount of SOD capable of inhibiting 50% of nitrite formation under assay condition. The enzyme activity is calculated from the value V_0 /V-1 (where V_0 is the absorbance of the control and V is the absorbance of the sample). At a time ten samples can be assayed for SOD activity by this method. In pilot experiments it is observed that in tubes where there is no Triton-X, the colour developed by nitrite with Greiss reagent has absorbance about 50% less than that of tubes having lowest concentration of Triton-X (final concentration in reaction mixture: 0.025%). However, further increase in Triton-X100 concentration (upto 3 fold) does not cause any significant change in colour formation. Methionine was used as an electron donor for reduction of riboflavin and it has no effect on Greiss reagent.

Greiss reagent was prepared freshly by mixing equal volume of 1% sulphanilamide in 5% phosphoric acid. L-Methionine and riboflavin were obtained from SRL, India while hydroxylamine HCl, N-1-napthyl ethylene diamine, sulphanilamide from Sarabhai M. Chemicals, India and Koch-Light Lab. Ltd., England, respectively. All reagents were prepared in double distilled water. They can be stored at 4°C and used for at least one week.

Effect of different concentrations of purified Cu-Zn SOD from bovine erythrocyte (Sigma Chemical Co., USA) on inhibition of nitrite formation from hydroxylamine by photo-reduction of riboflavin is depicted in Fig. 3. There is 50% inhibition of nitrite formation at 36 ng of pure SOD by the method reported here, whereas by cytochrome *c* method 50% inhibition is obtained by 200 ng of pure SOD; by NBT method 250 ng SOD; by xanthine: xanthine oxidase method 120 ng SOD and by nitrite method 60 ng is required. In comparison to other methods, in the present procedure about 5 ng pure SOD can be

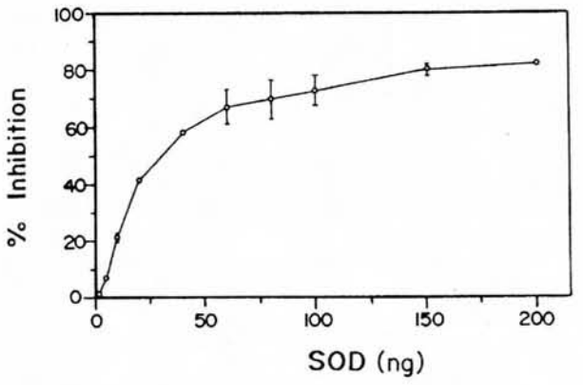

Fig. 3—Inhibition of nitrite formation by reaction of hydroxylamine with superoxide radical (generated by photoreduction of riboflavin) by purified bovine Cu-Zn SOD [50% inhibition of nitrite formation by Cu-Zn SOD is taken as one unit of enzyme activity. Data are expressed as mean ± S.D. of 6 observations]

detected which is far below the detection limit reported for many other methods. Activity of 5 μg pure SOD is inhibited to 95% by 2.5 m*M* KCN (Fig. 4). However, presence of KCN upto concentration of 5 m*M* in the assay system did not inhibit nitrite formation. Effect of different concentrations of partially purified SOD (acetone powder fraction followed by $(NH_4)_2SO_4$ precipitation prepared according to Crapo *et al.*[6]) from rat liver produced 50% inhibition at 600 ng protein concentration (Fig. 5). In Fig. 6, SOD activity of different concentrations of rat liver cytosol (10,000×*g* supernatant) is presented. A 50% inhibition is obtained at 40 μg protein concentration which is equivalent to 25 units/mg protein. On comparing the calibration curves for pure SOD, partially purified SOD and rat liver cytosol, it is found that not only the curves are identical but saturation level achieved by all the three preparations are similar (86-90%). Comparison of SOD activity in 10,000×*g* supernatants of liver, cerebral hemisphere and testis of Wistar rat by the three methods is presented in Table 2. The supernatants were passed through Sephadex G-25 column to remove interfering small molecules prior to assay.

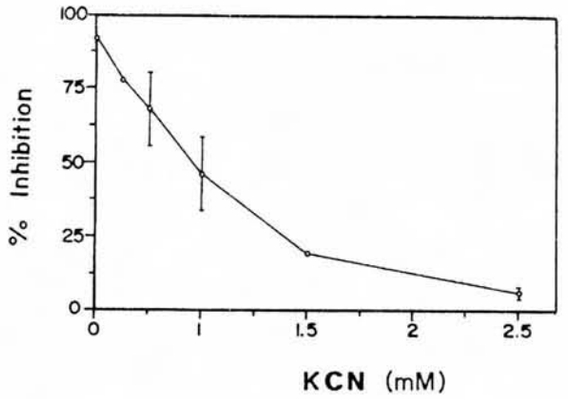

Fig. 4—Effect of different concentrations of KCN (m*M*) on partially purified rat liver SOD (5 μg) activity upon inhibition of nitrite formation by superoxide radical formation by photoreduction of riboflavin [Data are expressed as mean ± S.D. of 6 observations]

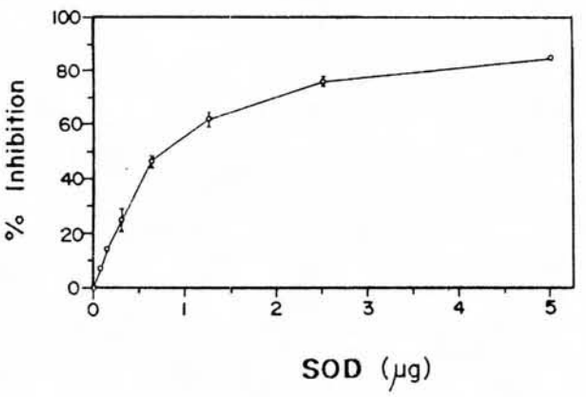

Fig. 5—Effect of different concentrations of partially purified rat liver SOD on inhibition of nitrite formation by superoxide radical generated from photoreduction of riboflavin [Data are expressed as mean ± S.D. of 6 observations]

The present method is two times less sensitive than the NADH method[4] but about ten times more sensitive than the NBT method[3]. Rat liver mitochondrial SOD activity is found to be 7.61±0.88 (n=5)

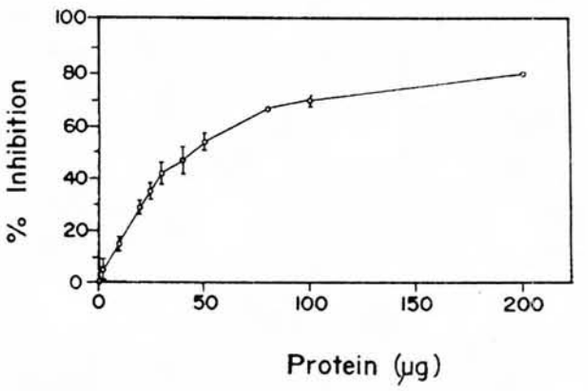

Fig. 6—Effect of different concentrations of rat liver 10,000×*g* supernatant on inhibition of nitrite formation by superoxide radical generated from photoreduction of riboflavin [The supernatants were passed through Sephadex G-25 column before assay. Data are expressed as mean ± S.D. of 6 observations]

Table 2—Comparison of SOD activity in 10,000 × *g* supernatants of different tissues of rat by three different methods [Units are expressed per mg of protein. Data are mean ± S.D. of 3 observations. Values in parentheses represent amount of protein]

Method	Liver		Brain		Testis	
	% Inhibition	Units	% Inhibition	Units	% Inhibition	Units
NBT (600 μg)	52.85 ± 5.47	1.83 ± 0.40	45.34 ± 3.28	1.40 ± 0.19	36.63 ± 2.95	0.98 ± 0.13
Nitrite (50 μg)	43.63 ± 5.77	15.75 ± 3.63	41.64 ± 2.82	14.36 ± 1.69	32.99 ± 2.66	10.06 ± 1.41
NADH (25 μg)	83.67 ± 0.58	205.87 ± 7.47	73.00 ± 0.00	109.03 ± 0.93	69.00 ± 0.00	89.00 ± 0.69

when estimated by the present method. This constitutes about 14% of the total SOD activity of rat liver. Measurement involves stable inexpensive reagents and can be adopted in any laboratory which has spectrophotometer/colorimeter and cold centrifuge facilities. Besides, many samples can be measured. The assay system can also be used to measure superoxide scavenging property of substances other than SOD.

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