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SHORT COMMUNICATION/KURZMITTEILUNG

Modification of the 6-Hydroxydopamine Technique for the Correct Determination of Superoxide Dismutase

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Summary: *Heikkila & Cabbat* (Anal. Biochem. *75*, 356–362 (1976)) have proposed the autoxidation of the 6-hydroxydopamine as a method to test superoxide dismutase activity in biological samples. This method has several advantages but in some instances leads to incorrect determinations. We present here a necessary modification of the method to avoid bias.

Introduction

Superoxide dismutase (EC 1.15.1.1) is an ubiquitous enzyme which catalyses the dismutation of the superoxide radical (O_2^-) to hydrogen peroxide (H_2O_2) and O_2. Determination of this activity has become a current practice since the discovery of the role of free radicals in a large number of pathological conditions such as inflammation, radiation injury, ischemia, aging, neoplastic processes, and syndromes due to chromosomal abnormalities.

In the current method for the determination of superoxide dismutase, O_2^- (generated enzymatically or nonenzymatically) is allowed to oxidize an indicator, and this reaction is monitored spectrophotometrically from the resulting absorbancy change. Superoxide dismutase destroys O_2^- and thereby decreases the initial rate of the absorbancy change of the indicator. This reduction in rate is a measure of superoxide dismutase activity. *Heikkila & Cabbat* have proposed autoxidation of 6-hydroxydopamine as a convenient and sensitive method for testing superoxide dismutase activity in biological extracts. The advantages of this method are:

a) the pH (7.4) used in the assay makes it possible to measure both Mn superoxide dismutase, inactive at pH higher than 9, and CuZn superoxide dismutase;
b) the large absorbance increase of oxidized 6-hydroxydopamine, an advantage particularly relevant in tissues where the superoxide dismutase content is relatively low;
c) the assay is rapid;
d) it is possible to use CN^- in the system to distinguish the CuZn superoxide dismutase activity from the Mn superoxide dismutase activity.

We noted, however that this method leads to erroneous superoxide dismutase determination in all biological samples containing low amounts of catalase. We present here a modification of the *Heikkila & Cabbat* method, which gives accurate results.

Methods

Reagents

6-Hydroxydopamine and pure bovine blood superoxide dismutase were products of Sigma. Catalase (65000 U/mg, free of superoxide dismutase activity in accordance with *Halliwell* (2)) was from Boehringer Mannheim. All other chemicals were analytical grade and were obtained from our usual commercial source.

Sample preparation

Human fibroblasts were cultivated in vitro from skin explants. They were sonicated in 20 mmol/l Tris/40 mmol/l KCl at pH 7.4 with Triton X 100 at a final concentration of 10 g/l and the suspension was centrifuged at 110000 *g* for 30 min. Protein was determined with the Biorad Protein Assay using bovine serum albumin as standard.

Human red cells, from heparinized venous blood, were washed twice in saline and haemolysed with 4 volumes of distilled water. To eliminate haemoglobin 0.4 ml of ethanol/chloroform (6.25/3.25, by vol.) was added to each ml of haemolysate and, after shaking, the suspension was centrifuged at 27000 *g* for 30 min. Haemoglobin was measured with the *Drabkin* method (Boehringer Cyanmethaemoglobin Standard Test).

Superoxide dismutase assay

We used the *Heikkila & Cabbat* method (1). Final concentrations in the reaction medium were 50 mmol/l sodium phosphate buffer, pH 7.4, 100 μmol/l EDTA, and 160 μmol/l 6-hydroxydopamine, prepared in 1 mmol/l KCl, pH 2.0, to avoid oxidation. We added 4.67 mg/l of catalase to the experimental sample, and the assay was run at 37 °C.

Statistical analysis

Student's t-test was used to compare the observed average percentage increment, either positive or negative, with an arbitrarily fixed ± 10%, which was chosen as the maximal increment over the basal value that could be due to experimental error.

Results

The effect of catalase on the rate of production of *p*-benzoquinone was tested during the linear phase of the autoxidation reaction; catalase decreased by 32% the rate of *p*-benzoquinone production of a 0.16 mmol/l 6-hydroxydopamine solution. In agreement with the results of *Sullivan* et al. (3), this effect is dose-dependent until a saturation point is reached. In our system saturation was reached at a catalase concentration of 4.67 mg/l.

Table 1 gives our results for the superoxide dismutase activity of different biological samples determined in the presence (experimental sample) and absence (control sample) of added catalase. The determined activities of pure bovine superoxide dismutase and the superoxide dismutase of in vitro fibroblasts are clearly increased by the presence of catalase, mean activities increasing by 25% and 45% respectively. No increase is seen, however, in the red cell lysate deprived of haemoglobin. In order to understand this difference we measured the endogenous catalase (4) in both extracts: the in vitro fibroblasts have an average (n = 5) value of 10.95 ± 1.44 and red cell lysates an average (n = 10) value of 111.0 ± 12.76 kU/g protein which is ten times greater. It is therefore concluded that the endogenous catalase of the haemolysate is sufficient to saturate the 6-hydroxydopamine present.

Tab. 1. Effect of catalase in the 6-hydroxydopamine assay for superoxide dismutase activity. Mean of kU/g protein ± S.E.; ns = not significant; *0.05 > p > 0.02; **p < 0.01

Sample	Superoxide dismutase activity		Increase
	basal	+ catalase 4.67 mg/l	(%)
Bovine superoxide dismutase (Sigma) n = 10	2584 ± 114	3237 ± 122	+ 25*
In vitro fibroblasts n = 5	34.52 ± 9.50	49.7 ± 15.9	+ 45*
Red cell lysates n = 10	2.76 ± 0.25	3.51 ± 0.31	+ 27**
Red cell lysates without haemoglobin n = 10	3.87 ± 0.26	3.68 ± 0.19	ns

We also tested the activity of bovine superoxide dismutase (n = 10) in the presence of 100 µmol/l azide and obtained an average value of 2003 ± 68 kU/g protein which is 22% lower than the average value obtained in the absence of azide (2538 ± 114). The difference is statistically significant with $p < 0.05$.

To study the interference of haemoglobin in the assay system we finally measured superoxide dismutase activity in crude haemolysates and in haemolysates treated with chloroform/ethanol, which precipitates haemoglobin without affecting catalase activity. In the haemolysates without haemoglobin the superoxide dismutase activity appears significantly higher (27%, $p < 0.01$) than in the crude haemolysate.

Discussion

According to *Sullivan* et al. (3), autoxidation of 6-hydroxydopamine produces H_2O_2 as well as O_2^-, and the peroxide would contribute to the production of coloured *p*-benzoquinone. Consequently, the presence of catalase can be expected to increase the inhibitory effect of superoxide dismutase in the assay. Nearly all biological samples contain endogenous catalase, but in different amounts, and in some cases the saturation point of the 6-hydroxydopamine present in the assay is not reached. In this case the determination of superoxide dismutase is inaccurate. The data presented in the table clearly illustrates this point.

Sullivan et al. (3) have suggested the use of azide, an inhibitor of catalase, to standardize the conditions of superoxide dismutase activity assay from tissue extracts. However the amount of azide needed to inhibit the whole catalase activity of any extract is of the order of 100 µmol/l. According to our data (see results) this amount also inhibits the superoxide dismutase enzyme, thus leading to an incorrect determination.

We have also noticed that the measurement of superoxide dismutase activity in crude haemolysates without the addition of catalase is not reliable, even though the activity of endogenous catalase may be high. A reasonable explanation of these results is the large production of H_2O_2 by the haemoglobin (5–7). We therefore consider it preferable to eliminate the haemoglobin. In our experience the epinephrine method (8) for the determination of erythrocyte superoxide dismutase activity is more precise and less influenced by interfering factors. In conclusion, when 6-hydroxydopamine is used for the assay, it is important to standardize the procedure by adding sufficient catalase to saturate the 6-hydroxydopamine, particularly if different tissues are to be compared with unknown endogenous catalase activities.

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