A Simple Sensitive Superoxide Dismutase (SOD) Assay and Its Serum Levels among Various Pathological Conditions

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SUMMARY A simple and sensitive assay for serum superoxide dismutase (SOD) was devised by using a xanthine-xanthine oxidase system in suitable pH condition and with Triton X-100.

Respective coefficients of variation for within-run and day-to-day precision of 3.8% and 8.0% were obtained. The analytical recovery for sera supplemented with SOD was 96% and the normal range of serum SOD was 8.08±1.76 (mean±SD) U/ml.

Serum SOD activity were determined in adult patients of diabetes mellitus, hypertensive diseases, hepatitis, malignant tumors and liver-cirrhosis which seem to be attributable to oxygenic toxicity. Higher level of SOD was shown in several of these patients.

Introduction

Superoxide dismutase (EC 1.15.1.1. SOD) dismutes the superoxide to less toxic forms. The superoxide is produced by the single electron reduction of oxygen, and the injurious effects to living cells have been reported by many investigators13). Since SOD was first found in bovine erythrocytes by McCord and Fridovich in 19694), it has been recognized as a significant catalyst in superoxide degradation. There have been several reports about SOD activity in erythrocytes8-12) and tissues8-12), whereas no estimate of its activity in serum has yet been reported. We showed a sensitive SOD assay for serum by modifying the methods proposed by Ishiguro et al.13) and by Beauchamp and Fridovich14), the SOD assay is made up of a competitive reaction for superoxide consumption between the tetrazolium reduction to formazan and the decomposition of superoxide to O2 and H2O2 by function of SOD.

Materials and Methods

Subjects: Sera were collected from 20- through 69-year old male and female patients with diabetes mellitus, carcinoma, hypertension, acute hepatitis, and liver-cirrhosis. These diseases were confirmed...
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histopathologically. Sera were collected from the normal control group containing males and females between 24 and 58 years of age.

Reagents: All chemicals used were of the reagent grade. Triton X 100, neotetrazolium chloride, hypoxanthine, and formaldehyde were purchased from Wako Chemical Co. (Tokyo). Xanthine oxidase was obtained from Boehringer Mannheim GmbH (Mannheim 31, West Germany). Bilirubin was a Dade Bilirubin Control (Dade Reagent, Miami, FL 33152 USA), and SOD purified from bovine erythrocytes was purchased from Sigma Chemical Co. (St. Louis, MO 63178 USA).

Methods: The reagent mixture was constituted from 2 volumes of 0.05 mol/l EDTA, 1 volume of 160 g/l Triton X-100, 4 volumes of 0.8 mmol/l neotetrazolium chloride and 2 volumes of 40 mg/l xanthine oxidase. The reaction stopper was prepared with 50 volumes of 1 mol/l formate buffer (pH 3.5), 18 volumes of 100 g/l Triton X-100, 25 volumes of 400 g/l formaldehyde, and 200 volumes of distilled water (DW).

One ml of the reagent mixture, 50 µl of sample and of DW for blank, 0.2 ml of 2 mmol/l hypoxanthine, and 0.8 ml of DW were mixed and then immediately incubated in glass tubes at 37°C. After incubating for 20 min, 2 ml of the reaction stopper was added and the percentage of SOD activity as inhibition of neotetrazolium reduction was determined spectrophotometrically at 510 nm as follows:

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\text{SOD inhibition(%) = } \frac{A_{510nm \text{ in DW (blank)}} - A_{510nm \text{ in sample}}}{A_{510nm \text{ in DW (blank)}}} \times 100
\]

In the present system, one unit of SOD activity was defined as the amount of enzyme giving a 50% inhibition of the maximal reduction of neotetrazolium, in accordance with the method of Beauchamp and Fridovich14).

Results

Figure 1 shows the relationship between formazan formation and serum volume under two pH conditions in the same serum. On the condition of pH 10.2, SOD activity was 7-fold greater than that of the neutral buffer condition (pH 7.5). When sample volumes were increased in the assay system, inhibition percentage of neotetrazolium reduction was shown in Figure 2. The present system showed the maximal inhibition in the

Fig. 1 The relation between formazan formation and serum volumes under the two pH conditions in the same serum. Formazan formation was shown in two pH conditions; 0.05 mol/l Tris-HCl buffer (pH 7.5 ; ○——○) and 0.05mol/l sodium carbonate buffer (pH 10.2 ; △——△). The range was shown with the standard error of the mean.
Fig. 2 The relation between inhibition (%) of the neotetrazolium reduction and serum volume. The present system showed the maximal inhibition in the mean of 3 sera to be 84%. Therefore, one half (42%) of the maximal inhibition was defined as one unit of SOD activity. The range was shown with the standard error of the mean.

Fig. 3 Recovery test for SOD assay. This study used the standard which was defined as one unit in the method of McCord and Fridovich. Inhibition (%) of the standard SOD of 0.05, 0.1, and 0.2 U with (■—■) and without (●—●) serum are shown. When the serum SOD activity showed the 20% inhibition, standard SOD of 0.05, 0.1, and 0.2 U were added to the serum. The recovery (%) was estimated as the amount of SOD recovered from the serum/amount of SOD added, though the actual result was 96.0%.

Fig. 4 The interference of bilirubin to SOD assay. A downward trend of formazan formation in serum to which one g/1 of bilirubin had been added (---), compared to that without the addition of bilirubin (——) in spite of having its own bilirubin color at 490 nm. One g/1 of bilirubin aqueous solution is also shown (———).
was found to be negligible, because the total bilirubin concentration in serum was below 1.0 g/l, even in the case of hyperbilirubinemia.

SOD levels in each patient group were shown in Figure 5. The values (mean±SD) obtained were as follows: carcinoma; 11.3±6.7, hepatitis, 10.8±2.3, liver cirrhosis; 10.7±2.7, diabetes mellitus; 10.2±3.4, hypertension; 10.1±3.5, and normal control; 8.1±1.8 U/ml. The normal control group showed the significant low SOD activity in comparison with patient groups (T<0.01).

The levels in the patients with diabetes mellitus, carcinoma, hypertension, hepatitis, and liver-cirrhosis often proved to be higher than those of the normal control group.

Discussion

We enabled the serum SOD activity modifying by the method of Beauchamp and Fridovich14) in suitable pH condition and with Triton X-100 according to Ishiguro et al.13).

It has been reported that the SOD activity in placental extracts, held at -20 ºC for 8 years showed no decreases8), while erythrocyte SOD activity in blood had decreased by 0 to 30% after standing for 3 days at room temperature, though it could be stored at 4ºC for up to 10 days without measurable loss5). The results of the present study indicated that no significant change took place in serum SOD activity when the serum had been held for at least 2 months at -20ºC, and that serum SOD activity was the same as that of plasma.

There are several papers treating the relationship between disease and SOD activity except in serum5-7), although the present paper was an estimate of serum SOD activity.

SOD levels in patient groups with diabetes mellitus, carcinoma, hypertension, hepatitis, and liver-cirrhosis seem to be higher than that in a normal control group. In our unpublished report, we showed that the serum level of lipoperoxide (measured as malondialdehyde), which is produced by superoxide, was higher in some patients than the normal control (date were not shown). These results suggest that the excessive superoxide may induce the elevation of SOD activity as reported by Crapo et al.15) in rat experiments. Thus, SOD may function to suppress the increased production of lipoperoxides and
may prevent cell injury caused by excessive superoxide.

SOD exists in almost all living cells, and is especially abundant in the erythrocytes and in cells from the liver, adrenal gland, and kidney\textsuperscript{8,9}. SOD may gain access to the plasma from injured and even intact cells, as other intracellular enzymes. In the present study, several high levels were found in each patient group, but others showed an overlap in values between normal control and other patient groups.

Key words: new assay, superoxide dismutase, human serum, superoxide, enzyme.

References