# Spectrophotometric Flow Injection Analysis of Superoxide Dismutase

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**A novel flow injection analysis (FIA) for superoxide dismutase (SOD) activity was developed based on the use of tetrazolium salt XTT (3'-{1-[(phenylamino)-carbonyl]-3,4-tetrazolium}-bis(4-methoxy-6-nitro)benzenesulfonic acid hydrate) and an enzyme reactor packed with Sepharose 4B on which xanthine oxidase (XO) and catalase were co-immobilized. No adhesion of XTT formazan to FIA line was observed in a continuous operation for 1 month, meaning that XTT formazan has enough solubility to the application to the FIA. Under the optimized conditions, the concentration of SOD giving 50% inhibition was 70 μg/ml and it was possible to detect 20 ng of SOD preparation in one sample. The relative standard deviation (n=10) was better than 1% and the sampling frequency was about 30 samples/h. The enzyme reactor showed no change in the response by 140 repetitive analyses.**

Keywords: Superoxide dismutase, tetrazolium, XTT, xanthine oxidase, FIA.

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Antioxidant enzymes are of vital importance in an organism's defense against oxidative stress. The most important are SODs (EC 1.15.1.1) which catalyze the dismutation of superoxide anion into hydrogen peroxide and molecular oxygen.[1,2]

Since the discovery of SOD,[3] numerous direct and indirect methods for the assay of SOD have been developed.[4] Compared with direct methods, indirect methods are more commonly used due to the convenience. Typical indirect methods involve enzymatic generation of the superoxide anion in the assay medium and competition between the superoxide scavenger and the SOD-catalyzed dismutation of superoxide. The most commonly used combination of the latter example is XO/ nitroblue tetrazolium salt (NBT).[5] However, NBT forms mono and diformazan that are only sparingly soluble in water when it is reduced. This property is not suitable for a precise assay of SOD. The direct interaction between NBT and the reduced form of XO is also known to occur.[6] Recently, we proposed a novel assay method of SOD using a tetrazolium salt XTT and reported that the method permitted to perfectly overcome those drawbacks which the conventional NBT method had.[7] Here, in order to increase the rapidity of the XTT method, we developed an FIA system based on the assay method.

The principle of the present method is shown in Fig. 1. The immobilized XO in a reactor was used for the generation of superoxide anion and hypoxanthine was selected as the substrate. In the absence of SOD, a maximum amount of XTT reduced with superoxide anion is observed (Control). The presence of SOD suppresses the formation of the reduced XTT. Therefore, the suppression ratio against the Control can be regarded as the inhibition ratio of each sample.

## Experimental

*Chemicals.* SOD (EC 1.15.1.1; 4000 units/mg protein) from bovine erythrocytes and catalase (EC 1.11.1.6; 41000 units/mg protein) from bovine liver were purchased from Sigma Chemical Co. (St. Louis, USA). XO (EC 1.2.3.2; 0.38 units/mg) from butter milk, NBT and XTT were obtained from Oriental Yeast Co. (Tokyo, Japan), Wako Pure Chemical Industries (Osaka, Japan) and Polyscience (Warrington, USA), respectively. CNBr-activated Sepharose 4B was purchased from Pharmacia LKB (Uppsala, Sweden). All other chemicals were of analytical reagent grade and were

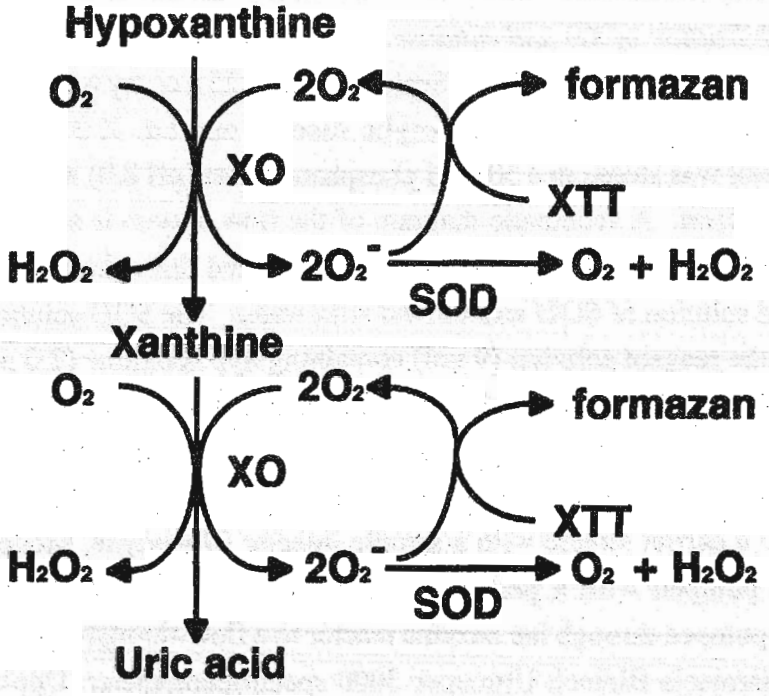



**Fig. 1** Principle of the present SOD assay

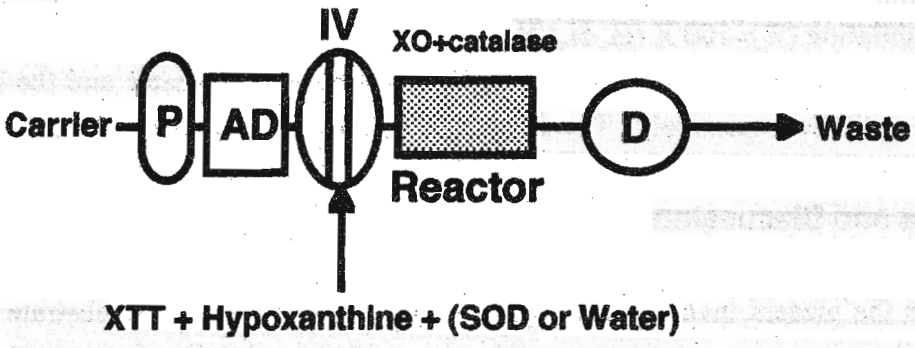



**Fig. 2** FIA manifold for SOD assay. P, pump; AD, air damper; IV, injection valve; D, detector.

used without further purification. All solutions were prepared with water purified by a Mill Q-system (Millipore). XTT was dissolved into the buffer at 50°C.

*Immobilization of XO and catalase.* XO (1.9 units) and catalase (34440 units) were immobilized on CNBr-activated Sepharose 4B (0.25 g of dry weight), as previously reported,[8] and was packed into an acrylic tube (2 mm i.d. X 3.7 cm long). The enzyme reactor was stored in a 50 mM phosphate buffer (pH 8.0) at 5°C.

*Flow system.* A schematic diagram of the flow system is shown in Fig. 2. A Teflon tube (0.86 mm i.d. and 1.4 mm o.d.) was used throughout the flow system. The standard solution of SOD was diluted with water. The SOD solution (1 vol) was mixed with the reagent solution (9 vol) containing hypoxanthine (2.0 mM) and XTT (2.3 mM), unless otherwise mentioned. The reagent solution was prepared with the carrier solution. At the experiments on the optimization, water was mixed with the reagent solution instead of the SOD solution. The mixed solution (20 $\mu$l) was injected into a carrier stream with a sample injector (Rheodyne, Model 9125). The carrier was pumped with a peristaltic pump (Gilson, Miniplus 3). The injected sample was pumped through the enzyme reactor to a flow-through spectrophotometric detector (Pharmacia Biotech Ultrospec 3000 spectrophotometer; Uppsala, Sweden). The formation of the reduced XTT formazan was monitored with an absorbance change at 470 nm. The inhibition ratio (%) of each sample was calculated as follows:

$$\text{Inhibition (\%)} = 100 \times (H_0 - H_1)/H_0$$

where $H_0$ and $H_1$ represent the peak height observed at the Control and the sample containing SOD, respectively. The determinations were carried out at 25°C.

## Results and Discussion

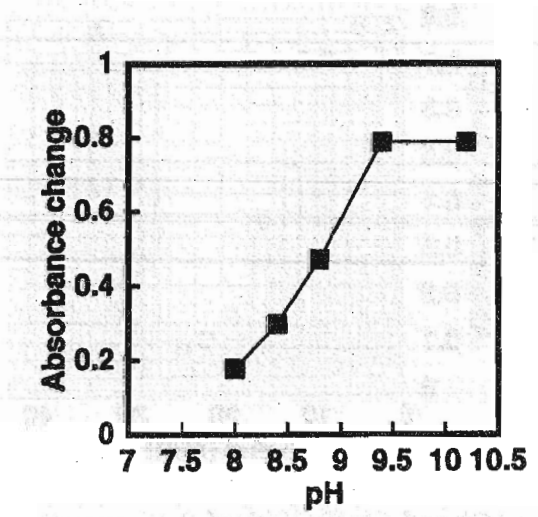
In the present investigation, hypoxanthine was selected as a substrate of XO from following reasons; (1) hypoxanthine has a higher solubility than xanthine and (2) the stability of the enzyme reactor was higher in hypoxanthine than xanthine.

At the beginning of the experiment, an enzyme reactor in which only XO was immobilized was used. When the reagent solution containing no SOD was injected repeatedly into the enzyme reactor, the peak response gradually decreased with the injection number. When catalase was immobilized together with XO, the stability

was improved depending on the added amount of catalase. This result suggests that hydrogen peroxide generated in the oxidation of hypoxanthine is partly responsible for the inactivation of XO. Based on a higher stability and response, 1.9 units of XO and 34440 units of catalase were co-immobilized on CNBr-activated Sepharose 4B (0.25 g of dry weight).

The effect of reaction pH on the response was examined in the pH range 8.0-10.2 (Fig. 3). The response rapidly increased with raising pH and a maximum response was obtained in the range pH 9.4-10.2. A similar pH dependence was found in luminescence studies on XO by Totter *et al.*[9]

The response linearly increased with raising the XTT concentration. Because a longer time was required in the preparation of a higher concentration, the concentration of XTT was set to 2.3 mM. Figure 4 depicts the effect of the concentration of hypoxanthine on the response. The response increased with an increase in the concentration and leveled off beyond 2 mM. From this result, 2 mM hypoxanthine



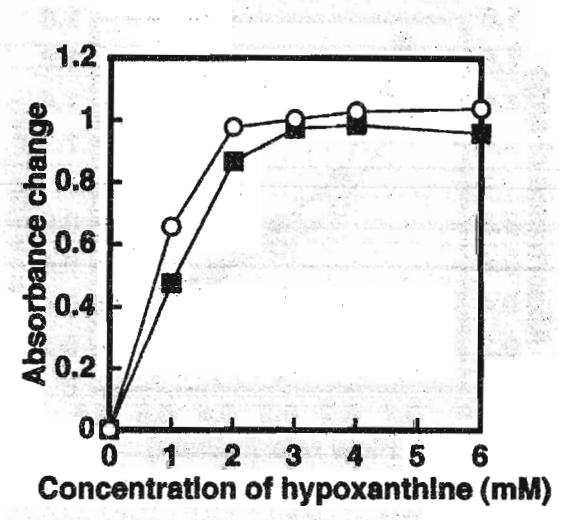



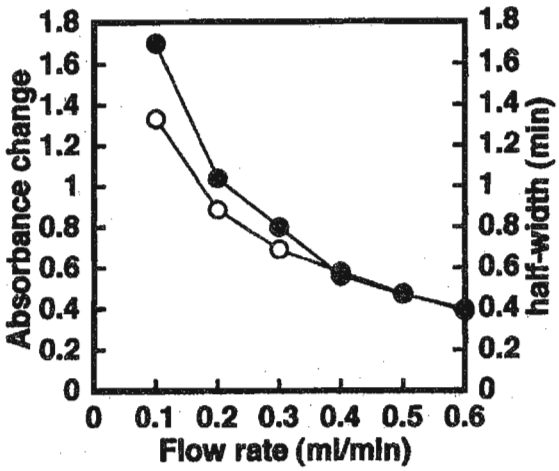

**Fig. 3** Effect of carrier pH on the response. The experimental conditions were as follows: carrier solution, 50 mM carbonate buffer (pH 9.4 and 10.2) or 50 mM phosphate buffer (pH 7.0, 7.5 and 8.0); hypoxanthine concentration, 2 mM; XTT concentration, 2.3 mM; flow rate, 0.4 ml/min.

**Fig. 4** Effect of hypoxanthine concentration on the response. The experimental conditions were as follows: carrier solution, 50 mM carbonate buffer (pH 9.4, ■; pH 10.2, ○); XTT concentration, 2.3 mM; flow rate, 0.4 ml/min.

was used in a subsequent experiment.

When the flow rate was changed in the range 0.1 to 0.6 ml/min, both the response current and the time for a baseline reversion decreased along with an increase in the flow rate (Fig. 5). The choice of the flow rate involves a compromise between the sensitivity and the sampling rate. A flow rate of 0.4 ml/min was used in a subsequent experiment, considering the relatively high response and short sample output time.

Under the optimized conditions, SOD activity was determined (Fig. 6). As the concentration of SOD increased, the peak height of each response decreased. Figure 7 shows the inhibition curve at each pH. When the excessive amount of SOD was injected, the inhibition ratio approached to almost 100% as was expected.[8] At pH 10, the concentration of SOD giving 50% inhibition ($IC_{50}$) was 70 $\mu$g/ml and it was possible to detect 10 $\mu$g/ml as a 10% inhibition level. The detection level corresponded



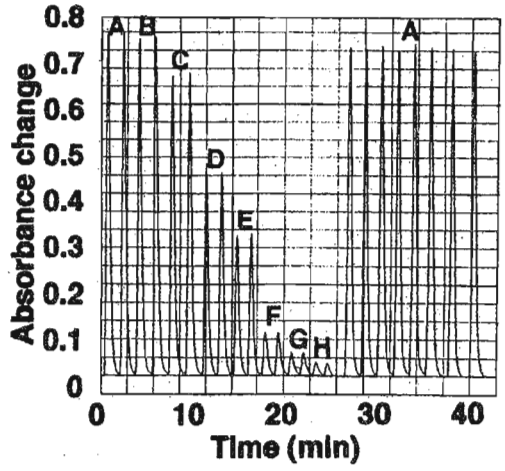




**Fig. 5** Effect of flow rate on the response (○) and a half-width of the response (●). The experimental conditions were as follows: carrier solution, 50 mM carbonate buffer (pH 10.2); hypoxanthine concentration, 2.0 mM; XTT concentration, 2.3 mM.

**Fig. 6** A typical response curve of SOD preparation. The used flow rate was 0.4 ml/min and other conditions were as same as those in Fig. 5. A, 0 $\mu$g/ml; B, 1 $\mu$g/ml; C, 10 $\mu$g/ml; D, 50 $\mu$g/ml; E, 100 $\mu$g/ml; F, 500 $\mu$g/ml; G, 1000 $\mu$g/ml; H, 2000 $\mu$g/ml.

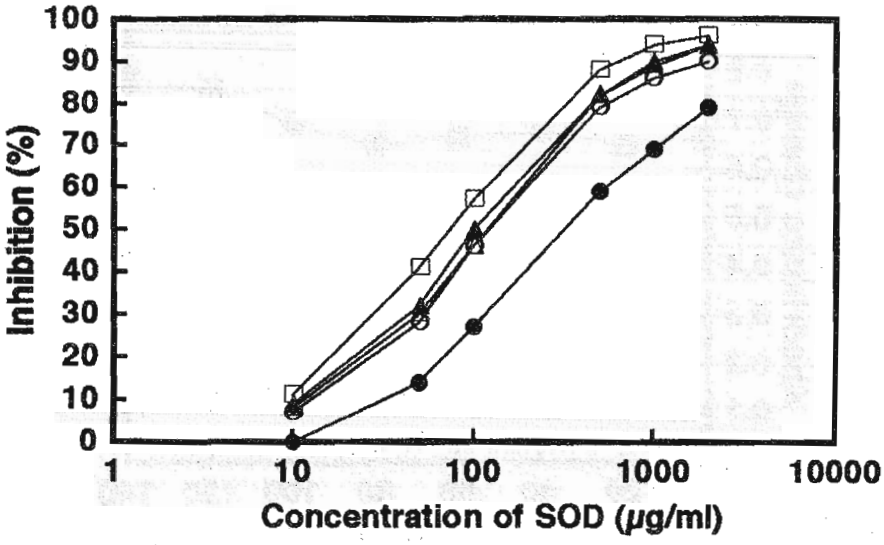



**Fig. 7** SOD inhibition curves at various pHs. pH 8.0, ●; pH 8.4, ○; pH 8.8, ▲; pH 9.4, △; pH 10.2, □. The experimental conditions were as same as those in Fig. 6.

to 20 ng as the amount per sample. Although the $IC_{50}$ became larger with a decrease in pH, this tendency was also same as that of the batch method.[7]

The operational stability of the immobilized enzyme was estimated by 140 repetitive injections (Fig. 8). No tendency of the response to decrease was recognized within the use. When the enzyme reactor was stored at 5°C for 3 months, no change of the response was recognized. Therefore, the reactor was suggested to have relatively high operational and storage stability. The relative standard deviation for ten successive injections of only reagent solution was better than 1% and the sampling frequency was about 30 samples/h. No adhesion of the XTT formazan to the FIA line and the enzyme reactor was observed at all even in a continuous operation for 1 month. This means that the XTT formazan has enough solubility to the application to the FIA.

The conventional spectrophotometric assay of SOD using tetrazolium salts requires a longer time than 20 min per sample. The FIA assay proposed in the present investigation requires only 2 min for one sample. Therefore, we believe that the rapidity of the present FIA method can liberate many biochemists from tedious and complicated experimental procedures required for assay of SOD.

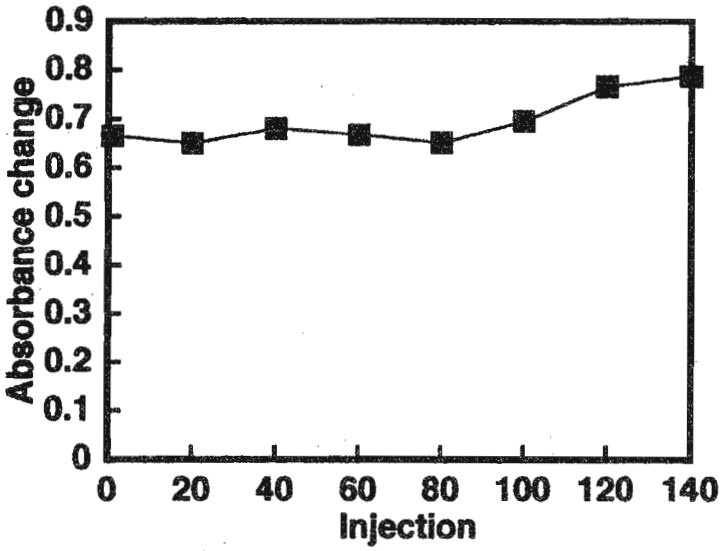



**Fig. 8** Operational stability of enzyme reactor. The reagent solution containing no SOD was injected under the conditions described in Fig. 6.

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