Spectrophotometric Assay for Superoxide Dismutase Based on the Reduction of Highly Water-soluble Tetrazolium Salts by Xanthine-Xanthine Oxidase

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Two novel highly water-soluble tetrazolium salts, WST-1 (4-[3-[4-(iodophenyl)]-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate sodium salt) and WST-8 (4-[3-(2-methoxy-4-nitrophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate sodium salt) were applied to the assay of superoxide dismutase (SOD). The superoxide anion generated by xanthine/xanthine oxidase (XO) reduced WST-1 and WST-8 to water-soluble formazans which exhibited absorbance maxima at 438 and 460 nm, respectively. The rates of reduction were linearly related to the XO activity, and reduction was inhibited by SOD. Complete inhibition by SOD of the reduction of both WST-1 and WST-8 was achieved, suggesting that these WSTs were not reduced with XO. WST-1 was found more useful than WST-8 because it had shown higher sensitivity which was apparently not dependent on the assay pH value in the range pH 8.0–10.2. These properties of WST-1 are ideal for the spectrophotometric assay of SOD in an aqueous system.

Key words: superoxide dismutase; assay; water-soluble tetrazolium; xanthine oxidase; WST

Superoxide dismutase (SOD; EC 1.15.1.1), which catalyzes the dismutation of the superoxide anion into hydrogen peroxide and molecular oxygen, is one of the most important antioxidative enzymes.1,2) Since the discovery of SOD,3) numerous direct and indirect methods for the assay of SOD have been developed.4,5) Compared with direct methods, indirect methods are more commonly used due to their 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 xanthine oxidase (XO)/nitroblue tetrazolium salt (NBT). However, NBT forms mono- and di-formazans that are only sparingly soluble in water when reduced. This property is not suitable for a precise assay of SOD. A direct interaction between NBT and the reduced form of XO is also known to occur.6) Recently, a novel assay method for SOD, using the tetrazolium salt, XTT (3-[3-tetrazolium]-bis[4-methoxy-6-nitro]benzenesulfonic acid hydrate), was developed by our research group.7) This assay method completely overcomes those drawbacks of the conventional NBT method.7) Although the XTT assay can be thus regarded as one of the most ideal assay methods for SOD among those developed so far, there are still some disadvantages with this assay. The limited solubility of XTT that required heating at about 50°C in order to prepare the optimum concentration of XTT (0.75 mM) and its pH dependence on the SOD assay, in which IC50 increased with reducing assay pH value, were disadvantages of XTT.7)

We apply here two kinds of highly water-soluble tetrazolium salts, WST-1 and WST-8 (Fig. 1), to the SOD assay in order to overcome the drawbacks in using XTT. These tetrazolium salts, with a corresponding highly water-soluble formazan, have recently been synthesized by Ishiyama et al.8,9) and appeared promising in clinical analyses for detecting NADH or the NADH-dependent enzyme (dehydrogenase) assay.8,9)

Materials and Methods

Reagents. WST-1 and WST-8 were kindly provided by Dojindo Laboratories (Kumamoto, Japan). SOD (EC 1.15.1.1; 4000 units/mg of protein) from bovine erythrocytes, XO (EC 1.2.3.2; 0.3 units/mg) from buttermilk, NBT and XTT were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.), Oriental Yeast Co. (Tokyo, Japan), Wako Pure Chemical Industries (Osaka, Japan) and Polyscience (Warrington, U.S.A.), respectively. All other chemicals were of analytical reagent grade and were used without further purification. All solutions were prepared with water purified by a Milli-Q system (Millipore). XTT was dissolved in the buffer at 50°C.

SOD assay with the XO/WST system. Into 2.5 ml of a 50 mM sodium carbonate buffer (pH 9.4 and 10.2) or sodium phosphate buffer (pH 7.0 and 8.0), 0.1 ml of 3 mM xanthine, 3 mM EDTA, a WST solution and the sample solution containing SOD or water were added. The reaction was initiated by adding an XO solution (0.1 ml). The absorbance change at 438 nm (WST-1) or at 460 nm (WST-8) over 20 min was monitored with a Pharmacia Biotech Ultrospec 3000 spectrophotometer maintained at 25°C.

SOD assay with the XO/XTT system.7) Into 2.5 ml of a 50 mM sodium carbonate buffer (pH 10.2), 0.1 ml of 3 mM xanthine, 3 mM EDTA, a 0.75 mM XTT solution and the sample solution containing SOD were added,

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The reaction was initiated by adding 56 mU/ml of an XO solution (0.1 ml). The absorbance change at 470 nm over 20 min was monitored at 25°C.

\textit{SOD assay with the XO/NBT system.}\textsuperscript{12) Into 2.4 ml of a 50 mM sodium carbonate buffer (pH 10.2), 0.1 ml of 3 mM xanthine, 3 mM EDTA, 0.75 mM NBT, 15% bovine serum albumin and an SOD solution or water were added. The reaction was initiated by adding XO. The absorbance change at 560 nm was monitored at 25°C for 20 min.

\textit{SOD determination of rat blood.} SOD was prepared from erythrocytes of fresh rat blood samples according to Maral \textit{et al.}\textsuperscript{19} with a slight modification. One unit of SOD activity is defined as the amount of the enzyme causing 50% inhibition of the reduction of each formazan observed in the blank.

\section*{Results and Discussion}

WST-1 and WST-8 were highly water-soluble, having a solubility greater than 100 mM, while the solubility of XTT was less than 2 mM.\textsuperscript{5,6) Heating was therefore not required in preparing a concentration of 1.5 mM, the maximum concentration of WST-1 and WST-8 that was used in the present investigation. Upon reduction with the superoxide anion, the reduced forms of WST-1 and WST-8 showed absorption maxima at 438 nm and 460 nm, respectively. The yellowish compounds were very stable at room temperature.

The effects of the XO activity, concentration of WST-1 and WST-8, and pH value on the reduction rate of WST-1 and WST-8 were investigated. Figure 2 shows the absorbance change over 20 min as a function of XO activity. This reaction time was selected because of a compromise between satisfactory reproducibility and rapidity of the assay. The absorbance increased almost linearly with increasing XO activity in the range lower than 116 mU/ml. At higher XO activity, in the time-course of the absorbance change, no linear change was observed over a reaction time of 20 min. As a consequence, the absorbance at an activity of XO higher than 116 mU/ml tended to level off. As shown in Fig. 3, the absorbance change increased with increasing WST-1 and WST-8 concentrations up to 0.4 mM. Beyond this concentration, the absorbance change reached a plateau where the reaction rate was too high to show a linear absorbance change over the reaction time of 20 min. When the concentrations of XO and XTT were set at 58 mU/ml and 0.75 mM, respectively, the absorbance linearly changed with reaction time up to 30 min, the absorbance change over 20 min being about 0.4–0.5. Figure 4 indicates the effect of the reaction pH on the absorbance change. The maximum change was recognized at pH 9.4 in both WST-1 and WST-8. As the molar extinction coefficient of WST-1 and WST-8 is almost constant in a pH range lower than 10,\textsuperscript{6,8) this pH dependency was due to the following three factors: the rate of superoxide anion formation, the rate of spontaneous superoxide anion dismutation, and the reactivity of each WST with the superoxide anion. This result is similar to the pH dependence of the XO/XTT system, at which the maxi-
mum absorbance change was observed at pH 9.4. Because both WST-1 and WST-8 showed an absorbance change greater than 0.25, even at pH 8.0, it was possible to perform the SOD assay in the pH range of 8–10.2. At pH 10.2, the addition of hydrogen peroxide (up to 10 mM as a final concentration) did not result in any formazan formation.

Using the optimized conditions, the activity of an SOD preparation was determined (Fig. 3). Complete inhibition was achieved with both the XO/WST-1 and WST-8 systems at all pH values examined. This result suggests that WST-1 and WST-8 were not directly reduced with the reduced form of XO. A similar result obtained with XTT has previously been reported and this property may thus be common with the water-soluble tetrazolium salts. With the XO/WST-1 system, there was no significant difference in IC50 value over the pH range. This result means that the sensitivity of the XO/WST-1 system did not depend on the assay pH value. At pH 10.2, the XO/WST-1 system showed higher sensitivity than the XO/WST-8 system by a factor of about 3. However, the XO/WST-8 system showed higher sensitivity with decreasing assay pH, there being no marked difference in the sensitivity between them at pH 8.0. This pH dependence of WST-8 was the reverse of that of XTT. Table 1 depicts IC50

![Fig. 3](image-url) Effect of WST-1 and WST-8 Concentration on the Reduction Process.

The reaction mixture contained 2.5 ml of a 50 mM carbonate buffer (pH 10.2) and 0.1 ml of 3 mM EDTA, 3 mM xanthine, 58 mU/ml of XO, and WST-1 (●) or WST-8 (○) having the concentration indicated on the abscissa.

![Fig. 4](image-url) Effect of Assay pH Value on the Reduction of WST-1 and WST-8.

The reaction mixture contained 2.5 ml of a 50 mM carbonate buffer (pH 9.4 or 10.2) or 50 mM phosphate buffer (pH 7.0 or 8.0) and 0.1 ml of 3 mM EDTA, 3 mM xanthine, 58 mU/ml of XO and 0.75 mM WST-1 (●) or WST-8 (○).

![Fig. 5](image-url) SOD Inhibition Curves Using the XO/WST-1 (●) and WST-8 (○) Systems.

The reaction mixture contained 2.5 ml of a 50 mM phosphate buffer (A, pH 8.0) or 50 mM carbonate buffer (B, pH 9.4; C, pH 10.2) and 0.1 ml of 3 mM EDTA, 3 mM xanthine, 58 mU/ml of XO, 0.75 mM WST and the sample solution containing SOD at the concentration shown on the abscissa.
values for four kinds of tetrazolium salts with the same SOD preparation at pH 10.2. WST-1 clearly indicates the highest sensitivity among them.

Using the above-mentioned optimized conditions, SOD activity in the erythrocytes of rats was determined, and compared with that obtained by the XO/XTT system. A linear relationship between WST-1 and XTT (r=0.968, n=7) and between WST-8 and XTT (r=0.994, n=7) was recognized. The slope of the regression curve almost reflected the difference in sensitivity between them. This result indicates that the XO/WST-1 and WST-8 systems were applicable to practical biochemical samples.

The XO/XTT system previously developed certainly improved on the conventional XO/NBT system for the following reasons: (1) XTT forms a water-soluble formazan; (2) no direct interaction was apparent between XTT and XO.7 Both the XO/WST-1 and WST-8 systems developed here also had those properties, meaning that these novel systems are similarly superior to the XO/NBT system. In addition, WST-1 and WST-8 are much more water-soluble than XTT. These characteristics indicate that both WST-1 and WST-8 would be more feasible than XTT for application to the SOD assay. In a comparison between WST-1 and WST-8, it can be concluded that the XO/WST-1 system would be more useful than the XO/WST-8 system because the former has higher sensitivity which is apparently independent of pH in the assay pH range of 8.0–10.2. The latter property means that the XO/WST-1 system is superior to the XO/XTT system. Therefore, the XO/WST-1 system could be the most convenient spectrophotometric method for assaying SOD activity in an aqueous system.

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References