Trace Determination of Superoxide Dismutase by Anodic Scan Pulse Voltammetry

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This assay for superoxide dismutase (SOD) involves two electrode reactions: 1) the generation of a substrate (O_2^-) by a one-electron reduction of dissolved oxygen at a constant potential of -1.0 V in a pH 11.0 borate-carbonate buffer solution containing 2×10^{-4} mol/l EDTA and 2.4×10^{-4} mol/l triphenylphosphine oxide, and 2) the detection of a residual substrate, which results from an SOD-catalyzed reaction at -0.1 V by one-electron oxidation with anodic scan pulse voltammetry. By the use of a hanging mercury drop electrode (a static mercury drop electrode) and with a sequence-control technique, these reactions are so strictly controlled that an SOD as low as 10^{-12} mol/l can be determined within a short time (<30 s) under automatic operation.

Keywords Superoxide dismutase, anodic scan pulse voltammetry, sequence-controlled polarography

Superoxide dismutases (SODs), which catalyze the dismutation of O_2^- according to

and protect living organisms from damage formed in the reduction of molecular oxygen to H_2O , are currently of interest regarding many biochemical systems.¹⁻⁴

Assays for SOD can be classified into two groups, according to the methods for the formation and detection of the substrate (O_2^-). The first method, which has been used in many biochemical studies, can be called an indirect assay.^{2,5-9} In this method O_2^- is generated enzymatically and reacts with appropriate indicators, such as cytochrome *c* and tetrazolium salts, so that the concentration of SOD can be calculated from the change in the concentration of these indicators. In general, indirect assays are useful from 10^{-9} to 10^{-10} mol/l of SOD concentration.^{6,8} However, these assays are influenced by many interfering effects caused by other systems in biological materials. Furthermore, the complex enzymatic reactions used in these assays are seriously influenced by the impurity of reagents, the reaction time and the pH of solutions.⁸⁻¹⁰ The second method, the direct assay, is based on the electrochemical generation and detection of O_2^- at the surface of a mercury electrode in the presence of surface-active substances, such as triphenylphosphine oxide.¹¹⁻¹⁵ Contrary to the enzymatic procedures, the generation and

detection of a stable substrate can be so precisely regulated at the electrode surface that this assay suggests the possibility of measuring very small amounts of SOD.

With the aid of a sequence control technique, Yamaguchi *et al.*¹⁷ have constructed a versatile electro-analytical measuring system, a sequence-controlled polarograph. By this system, both the formation period and the surface area of an indicator electrode are so precisely controlled that numerous electrochemical measurements can be carried out automatically with good reproducibility and with a minimum consumption of mercury.

The behavior of dissolved oxygen and the reaction mechanisms in a direct assay of SOD at the surface of a mercury electrode have already been discussed.¹³⁻¹⁶ In this study we focused on the introduction of a simple and reliable procedure for an assay of trace amounts of SOD, in which a sequence-control technique was combined with pulse-voltammetric techniques, such as anodic scan pulse polarography (ASPP) or potential stepping voltammetry.

Experimental

Material, reagents and solution

Cu, Zn-superoxide dismutase (SOD, EC. 1.15.1.1) from bovine liver was donated by Prof. Asada of Kyoto University. A weighed amount of SOD was dissolved

in triply distilled water (a stock solution) and the concentration of SOD in the stock solution was measured according to the absorbance at 680 nm using a molar absorptivity of $237 \text{ (mol/l)}^{-1} \text{ cm}^{-1}$.¹⁸ The specific activity of the SOD was 3547 unit per mg by McCord and Fridovich.⁵ Triphenylphosphine oxide (TPO, Tokyo Kasei Co., Tokyo) and ethylenediamine tetraacetic acid disodium salt (EDTA, Dojin Kagaku Co., Kumamoto) were of reagent grade. An air-saturated borate-carbonate buffer solution (0.2 mol/l (M), pH 11.0) containing 2×10^{-4} M EDTA and 2.4×10^{-4} M TPO was used as a basal solution. All other chemicals used were highly pure commercial products (Wako Pure Chemical Ind. Ltd., Osaka).

Apparatus

The electrochemical system was assembled from four units: an electrode unit in which a static mercury drop electrode (SMDE, Princeton Apply Research Co.) was used as an indicator electrode, a potential-supply and current recording unit, a data-processing unit and a controlling unit. Details concerning the construction and application of this system were reported previously.¹⁷

All electrochemical measurements were carried out in an H-type glass cell thermostated at $25 \pm 0.1^\circ \text{C}$. All potentials were measured against a saturated calomel electrode (SCE).

Measurement

Two and a half milliliters of a basal solution was transferred into the cell, and an aliquot of the stock solution was syringed into the basal solution manually. The following procedures were performed automatically according to the programmed timing sequence. The test solution was mixed for 15 s and kept still for 15 s. Just after the birth of a mercury drop, an initial potential (E_i , usually -1.0 V) was applied to the electrode for a definite time (T_i , usually 9 s), which was followed by a potential scan by ASPP, or more conveniently, stepped by a potential stepping voltammetry into positive direction.

The current due to the oxidation of O_2^- was measured at -0.1 V and was converted to the concentration of SOD.

Results and Discussion

Typical current-potential curves recorded by ASPP in the absence and presence of 2×10^{-9} M SOD in the basal solution are given in Fig. 1. The decrease in the anodic current height within the potential range -0.3 to -0.1 V has been attributed to a partial consumption of O_2^- by the SOD-catalyzed reaction. In this procedure the generation of O_2^- at the surface of the SMDE at the potential of E_i (-0.8 V to -1.0 V) for a given period of time (T_i) is expressed as

Fig. 1 Anodic scan pulse polarograms at an SMDE. (A), the basal solution (pH 11.0) and (B), (A) + 2×10^{-9} M SOD. The substrate (O_2^-) was generated at the potential of -1.0 V for 9 s.

during this reaction time, part of the produced O_2^- is consumed in the bulk of the solution according to

so that when the potential of SMDE is rapidly scanned or stepped to -0.1 V, the oxidation current due to the remaining O_2^- is observed as

The anodic current based on Eq. (4) can be expressed as

$$I_a = \kappa [\text{O}_2^-]^0, \quad (5)$$

where κ is a proportionality constant and $[\text{O}_2^-]^0$ indicates the concentration of O_2^- in the vicinity of the electrode surface. According to theoretical considerations concerning ASPP^{15,19}, the relationship between $[\text{O}_2^-]^0$ and the concentration of O_2^- in a solution ($[\text{O}_2^-]^*$) is expressed as

$$[D/\pi T_i]^{1/2} [\text{O}_2^-]^* = \{\mu \kappa C_{\text{SOD}} + (D/\pi T_i)^{1/2}\} [\text{O}_2^-]^0, \quad (6)$$

where D is the diffusion coefficient of both O_2 and O_2^- (the equivalent D value for the two species is assumed), μ is the thickness of the reaction layer in which the SOD-catalyzed reaction occurs, and k is the rate constant of Eq. (3). Substituting Eq. (6) into Eq. (5), we obtain

$$I_a^0/I_a = 1 + (\pi T_i/D)^{1/2} \mu \kappa C_{\text{SOD}}, \quad (7)$$

where I_a^0 is the I_a value at $C_{\text{SOD}}=0$, which is given by $I_a^0 = \kappa [\text{O}_2^-]^*$. At various concentrations of SOD and changing the O_2^- -generation time (T_i), the anodic

Fig. 2 Plot of I_a^0/I_a at -0.1 V against C_{SOD} . T_i : A, 9.0; B, 5.0; C, 2.8; D, 1.6 s.

Fig. 3 Plot of ΔI_a at -0.1 V against C_{SOD} by ASPP.

current was recorded by ASPP. From the oxidation current at -0.1 V, the ratio of two currents (I_a^0/I_a) vs. C_{SOD} plots gave straight lines with a vertical intersection of 1.0 (Fig. 2); at constant concentrations of SOD, such as 6×10^{-11} and 23×10^{-11} M, the ratio I_a^0/I_a increased linearly with $T_i^{1/2}$, as predicted by Eq. (7).

From the view point of an SOD assay, Fig. 2 shows that concentrations on the order of 10^{-10} to 10^{-11} M can easily be measured by calculating the ratio (I_a^0/I_a). At very low concentrations, Eq. (7) can be rewritten as

$$\begin{aligned} \Delta I_a &= I_a^0 - I_a \\ &= (\pi T_i / D)^{1/2} \mu k I_a^0 C_{SOD}. \end{aligned} \quad (8)$$

Thus, the decrement in I_a is expected to be proportional to C_{SOD} . Decrements at -0.1 V in ASPP were recorded with a successive addition of an aliquot of the stock solution of SOD to the basal solution and automatic operation. The results are given in Fig. 3. Each point in this figure represents an average value

and a maximum deviation. The ΔI_a value increased linearly with an increase in C_{SOD} on the order of 10^{-12} M (correlation coefficient of 0.997), as predicted by Eq. (8).

The sensitivity of this method is one order of magnitude higher than that of conventional polarography or cyclic voltammetry using catalytic oxygen reduction current¹¹⁻¹⁴, and is comparable to that of the spectrophotometric method⁷, which shows the highest sensitivity hitherto reported. This method, which is a direct assay, appears to provide a convenient method for assays of trace amounts of SOD and details in clinical applications will be reported in the near future.

The authors thank Prof. K. Asada and Dr. M. Takahashi of the Research Institute for Food Science, Kyoto University, for their donation of SOD and relevant advice in measuring the specific activity of the enzyme.

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(Received June 15, 1989)

(Accepted July 1, 1989)