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Short Communication

Enzyme immunoassay by polarography — A preliminary study

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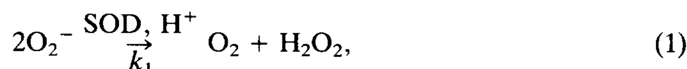
Introduction

The enzyme-linked immunoassay techniques (EIA), which use antigens labelled with enzymes, appear, in some cases, competitive with radioimmunoassay (RIA), since the sensitivity gap between these methods is narrowing. Furthermore RIA, which is typically used to analyse antigen concentrations in the range from 10^{-7} to 10^{-11}M, presents some disadvantages including a slight health hazard and a short reagent shelf-life.

In this paper we present some data on the utilization of the enzyme Cu, Zn superoxide dismutase (SOD) as an electroactive tag in homogeneous EIA tests. SOD is an intracellular enzyme and offers many advantages over other enzymes, especially because of its high activity which is:



$$2O_2^- \xrightarrow[k_1]{SOD,\ H^+} O_2 + H_2O_2, \qquad (1)$$

very close to the diffusion limit ($k_1 = 2.3 \times 10^9 M^{-1} s^{-1}$) [1, 2]. SOD is commercially available and has very high stability. A simple polarographic method, based on the catalytic currents [3] allows measurement of SOD concentrations as low as 10^{-11}M without presenting the background problems which affect the most sensitive voltammetric techniques such as differential pulse voltammetry and stripping voltammetry, when biological samples are analysed [4–7]. Using the catalytic currents method, SOD activity measurements may be carried out routinely in red blood cell lysates and cell homogenates [8]. The principle of the method is that molecular oxygen, in the presence of a surfactant such as triphenylphosphine oxide (TPO), is reduced at a dropping mercury electrode to superoxide ion. This then diffuses from the electrode surface into the reaction layer [9] where, in the presence of SOD, it partially changes into O_2 and H_2O_2 according to equation (1). The molecular oxygen generated in this reaction adds to

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the molecular oxygen diffusing from the solution to the electrode surface, with consequent increase of the polarographic current. The concentration of SOD in the solution can be related to the increase in the polarographic current by the equation:

$$7.42/[i_d/(i_l - i_d) - 1.25] = C + k_l tg\text{SOD}, \qquad (2)$$

where i_l and i_d are the limiting and the diffusion currents, respectively (see Fig. 1), tg is the drop time and C is a constant which takes into account the small fraction of O_2^- which changes spontaneously into H_2O_2 and O_2 in the reaction layer [10].

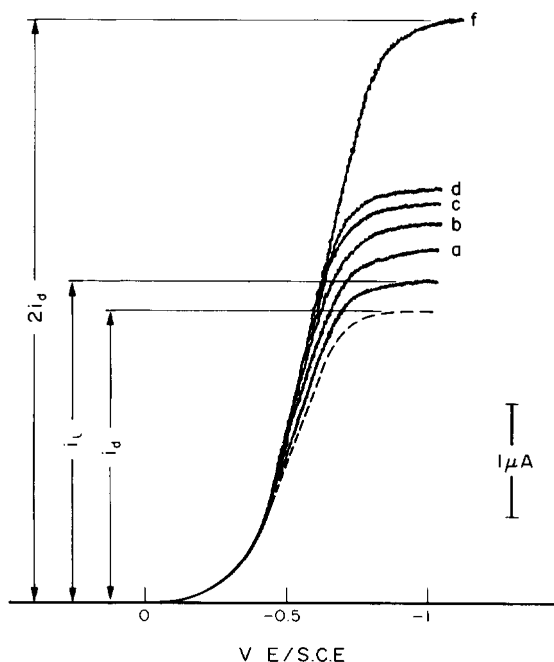

Figure 1
Polarographic measurements of the activity of DNP labelled superoxide dismutase (DNP–SOD). (a–d): O_2^- polarographic waves obtained by repetitive injections of four 40 μl aliquots of 2.5×10^{-8}M DNP–SOD solution incubated with DNP–Ag and ARGG before the addition in the polarographic cell. To obtain the $2i_d$ value, see polarogram f; the pH of the solution in the polarographic cell was brought into the range 4–5 by addition of 5 M CH_3COOH.

Experimental

Material

Rabbit antibodies specific for 2,4-dinitrophenyl labelled bovine serum albumin (DNP–Ab), titre 1.8 mg antibody/ml, and antirabbit gamma-globulin (ARGG), titre 2.65 mg antibody/ml, were supplied by Miles Laboratories Inc. (Elkhart, Ind.) and Biodata (Rome, Italy), respectively. *N*-ε-2,4-dinitrophenyl-L-lysine-HCl (DNP–Lys), Sigma (St. Louis, USA), was used as standard antigen, while bovine Cu, Zn superoxide dismutase was obtained from Miles.

Superoxide dismutase labelled with 2,4-dinitrophenyl (DNP–SOD) was made by adding 20 μl of 9 mM 1-fluoro-2,4-dinitrobenzene (DNFB) solution in anhydrous

acetone to 0.25 ml of an aqueous solution containing 7×10^{-5}M SOD and 0.1 M sodium borate (pH 8.2). The reaction mixture was kept at 22°C for 56 h; then the reaction was stopped by lowering the pH to 4.5 with acetic acid. The unreacted DNFB and the reaction by-products were removed by exhaustive dialysis. The UV spectrum of the SOD conjugate ($\epsilon = 1.6 \times 10^4 M^{-1} cm^{-1}$ at 351 nm) indicated an average of 4.8 DNP groups per SOD molecule. The final solution of 2,4-dinitrophenyl labelled SOD (DNP–SOD), 1.2×10^{-5}M in protein, was prepared by dilution with 0.02 M phosphate buffer (pH 7.1). The catalytic activity of the labelled SOD was 70% of that of the native enzyme and remained constant after months of storage at −20°C.

Procedure for displacement curves

Duplicate samples were prepared by mixing measured volumes (Micropettor pipettes) of various solutions in test tubes. Typically 200 μl of assay buffer (2×10^{-2}M sodium phosphate, pH 7.2, containing 0.1 mM EDTA, 9 mM sodium azide and 1 g/l gelatine) were transferred to each test tube, then 2 μl of 1.2×10^{-5}M DNP–SOD and 2 μl of DNP–Ab were added. After 5 minutes a concentrated DNP–Lys solution was added to obtain a DNP–Lys concentration in the range 10^{-8}–10^{-5}M, with a final volume of 210 μl. After 4 h of incubation at 22°C, 15 μl of ARGG were added to each test tube and the resulting solution was incubated for 4 h. The solution was tested for SOD activity, on an Amel 461 polarographic unit, according to the catalytic currents method [3, 8], drop time 3 s. Concentrations of free SOD in the range 5×10^{-11}–10^{-9}M were realized by addition of suitable aliquots of the solution under investigation in the polarographic cell containing 2 ml of 0.1 M sodium borate (pH 9.8). After each addition, the cell current was recorded as shown in Fig. 1. From the slope of the plot of the left hand side member of equation (2) versus the added volume (see Fig. 2) the concentration of the free enzyme was obtained according to equation (2).

Results and Discussion

The amount of free DNP–SOD, after incubation with rabbit antibodies specific for DNP, was calculated from the increase of the polarographic wave of O_2. This increase is due to the dismutation, by the free DNP–SOD, of the O_2^- generated at the electrode

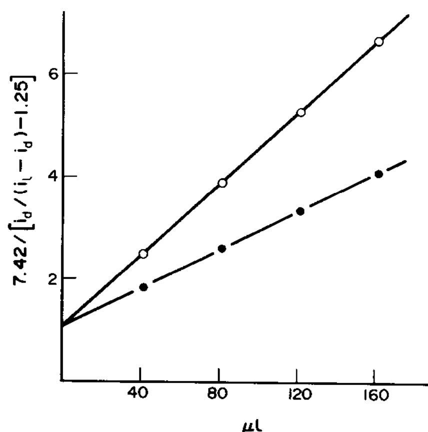

Figure 2
Plots of the left hand side of equation (2) versus the amount of DNP–SOD solution added in the polarographic cell. (○) 2.5×10^{-8}M DNP–SOD; (●) DNP–SOD incubated with DNP–Ab and ARGG before the addition in the polarographic cell.

surface, since the precipitated DNP–SOD–antibody complex does not contribute to the dismutation of the superoxide ion at the electrode surface. It was found that the polarographic measurements carried out with samples centrifuged at 2×10^3 **g** for two min to remove the precipitated DNP–SOD–antibody complex gave the same results within the experimental error and, as a consequence, the method we are proposing appears to be an homogeneous immunoassay test. The normalized activity values of solutions of DNP–SOD, after 4 h incubation with rabbit antibodies specific for DNP groups are reported in Table 1 (column 2) while the results of column 3 refer to the same experimental conditions plus ARGG. From the data of Table 1 it appears that the presence of ARGG, at DNP–SOD concentrations lower than 10^{-6}M, decreases the amount of the free DNP–SOD, and therefore it increases the response because of the more efficient precipitation of the DNP–SOD–antibody complex by ARGG. However, under our experimental conditions, a fraction of free DNP–SOD still seems to be present, as shown by the residual activity measured after addition of DNP–Ab and ARGG. From the data of Table 1 a calculation shows that, under our experimental conditions, the average association constant of the complex DNP–SOD–Ab is of the order of 10^8M^{-1}.

Table 1
Normalized activity of dinitrophenyl labelled Cu, Zn superoxide dismutase after incubation with dinitrophenyl specific antibodies

DNP–SOD ($M \times 10^7$)	Incubation with DNP–Ab	DNP–Ab and ARGG
30	0.401	0.404
10	0.410	0.413
5	0.473	0.425
1	0.532	0.473
0.3	0.641	0.532
0.1	0.719	0.605

Different concentrations of 2,4 dinitrophenyl labelled SOD (DNP–SOD) were incubated with DNP specific antibodies (DNP–Ab), being [DNP–SOD]/[DNP–Ab] = 1, in the presence and absence of 1.2×10^{-6}M antirabbit gammaglobulin (ARGG).
The activity of the solutions of DNP–SOD, before the addition of DNP–Ab, was taken as 1.

The curve of displacement of DNP–SOD from its complex with DNP–Ab by DNP–Lys is reported in Fig. 3. It will be seen that in the presence of 1.1×10^{-7}M DNP–SOD the displacement curve can be utilized to measure antigen concentrations in the range 10^{-8}–10^{-6}M.

The sensitivity of the polarographic method is apparent from Fig. 1, where the enhancement of the O_2^- polarographic wave due to 5×10^{-10}M increases in SOD concentration in the polarographic solution are shown. On this basis it appears that antigen concentrations of the order of 10^{-10}M can be easily measured using antigen–antibody systems characterized by stability constants higher than that of the system DNP–Ab which we have utilized.

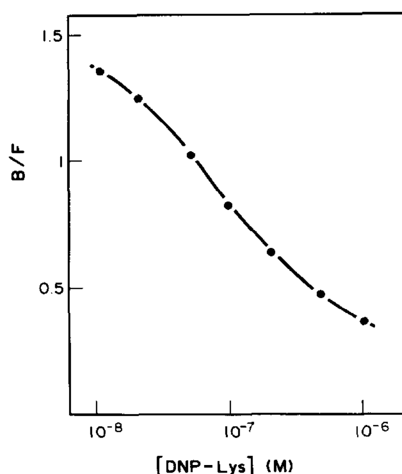

Figure 3
Antigen–antibody binding curve of DNP–SOD. Bound/free ratio (B/F) of SOD labelled DNP as a function of the concentration of the unlabelled DNP [DNP–Lys].

In was found that a matrix of human serum, urine and saliva did not disturb the polarographic wave. This is not the case with other electrochemical methods [4–7]. Considering the low detection limits, the convenience of homogeneous detection and the possibility of operating with biological samples, the method proposed appears promising for the detection of antigens such as drugs, hormones, etc. in biological fluids. Further advantages are the high stability of the Cu, Zn superoxide dismutase and its extremely high activity, making the cost of enzyme required for each test insignificant. Inexpensive and reliable instruments are commercially available.

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