Letters to the Editor

Sensitive Amperometry of 4-Aminophenol Based on Catalytic Current Involving Enzymatic Recycling with Diaphorase and Its Application to Alkaline Phosphatase Assay

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Alkaline phosphatase is a common enzyme used in electrochemical enzyme immunoassays, with phenyl phosphate as a substrate. The phenol compound produced by the enzyme-catalyzed hydrolysis of phenyl phosphate is measured by oxidative amperometry. Recently, the use of 4-aminophenyl phosphate as a substrate in these assays has been advocated and extensively studied by several authors.¹⁻⁶ In this note we report that 4-aminophenol, the hydrolysis product of 4-aminophenyl phosphate, can be amperometrically detected at concentration levels as low as 10^{-10} M ($M = \text{mol dm}^{-3}$) by the principle of polarographic catalytic current^{7,8}, in which enzymatic recycling⁹ with a redox enzyme (that is, diaphorase) is involved; the sensitivity of the electrochemical assay of alkaline phosphatase is greatly improved by this method.

Alkaline phosphatase (EC 3.1.3.1, Lot No. 06290) was purchased from Toyobo Co., Ltd. The enzyme was dissolved with a 1% bovine serum albumin (BSA) solution containing 0.25 mM MgCl_2 and stored in a refrigerator. Diaphorase (EC 1.6.99.—, Type I) from *Bacillus stearothermophilus* was a gift from Unitika Ltd. The enzyme was dissolved with a 1% BSA solution. 4-Aminophenyl phosphate (PAPP, Aldrich Chemical Co., Inc.), 4-aminophenol (PAP, Wako Pure Chemical Industries, Ltd.) and NADH (reduced form of nicotinamide adenine dinucleotide, Oriental Yeast Co., Ltd.) were used without further purification. PAPP was dissolved in a 0.1 M carbonate buffer (pH 10.3) containing 0.25 mM MgCl_2 , immediately before use. PAP was dissolved in a 0.02 M acetate buffer (pH 4.5). Other chemicals were of analytical-reagent grade.

The working electrode was a glassy carbon disk electrode ($\phi = 0.3$ cm). The electrode was polished to a mirror-like finish with 0.05 μm alumina, followed by through rinse with deionized water in an ultrasonic bath. The counter electrode was a platinum electrode. The potentials were measured against an Ag/AgCl (sat. KCl)

Fig. 1 Cyclic voltammograms of 4×10^{-4} M PAP in 0.1 M tris-buffer of pH 8.5 without (a) and with (b) 3.3×10^{-8} M diaphorase and 10^{-3} M NADH. Potential was scanned at 10 mV s^{-1} from -0.2 V.

reference electrode. Electrochemical measurements were carried out in an H-type plexiglass cell with a sample volume of 0.4 ml, thermostated at $37 \pm 0.3^\circ \text{C}$, with a potentiostat (Yanaco polarographic analyzer P-1000) connected with an X-Y recorder (Yokogawa Modal 3025).

Figure 1 shows cyclic voltammograms of 10^{-4} M PAP in a 0.1 M tris-buffer solution of pH 8.5 without (a) and with (b) 3.3×10^{-8} M diaphorase and 10^{-3} M NADH. The results clearly show that the anodic current due to the oxidation of PAP to 4-quinone imine (PQI),

is greatly amplified by enzymatic recycling:

Here, DIP(red) and DIP(ox) are the reduced and oxidized forms of diaphorase, respectively. Theoretical consideration shows^{7,8,10} that the (amplified) catalytic current, according to Eqs. (1)–(3), should increase linearly with the concentration of PAP, as well as with the square root of the concentration of diaphorase. These theoretical predictions were experimentally proved to be valid, giving the bimolecular reaction rate constant as $k_2 = 1.9 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ (pH 8.5, 25 °C) for Eq. (2).¹¹

Electrochemical determination of PAP

Calibration curves for PAP were prepared in order to determine the detection limits. Four microliters of the diaphorase solution (3.3×10^{-6} or $3.3 \times 10^{-4} \text{ M}$) and 4 μl of 0.1 M NADH were syringed successively into 382 μl of 0.1 M tris-buffer in an electrolysis cell to make the basal solution. An aliquot (usually 10 μl) of the standard solution of PAP was syringed in the basal solution, and the mixture was stirred with a magnetic rotor for a few seconds. The anodic current at +0.15 V was recorded. A stationary current response was observed immediately after each addition of the PAP solution. A linear calibration curve was confirmed for a concentration range of PAP between 5×10^{-9} and $1 \times 10^{-7} \text{ M}$ in the basal solution containing $3.3 \times 10^{-8} \text{ M}$ diaphorase. The relative standard deviation at $1 \times 10^{-8} \text{ M}$ PAP was 5.1% ($n=10$). Also, a linear calibration curve was confirmed for the concentration range of PAP between 1×10^{-9} and $1 \times 10^{-8} \text{ M}$ in the basal solution containing $3.3 \times 10^{-6} \text{ M}$ diaphorase with the slope of $4.6 \text{ nA} / 10^{-8} \text{ M PAP}$ and the noise level (base current) of 0.07 nA. The experimental detection limit defined as $3 \times \text{noise} / \text{slope of calibration curve}^6$ was $5 \times 10^{-10} \text{ M}$. This study is being continued so as to further improve the detection limit.

Alkaline phosphatase assay

One microliter of the alkaline phosphatase solution was syringed into 49 μl of a carbonate buffer (pH 10.3) containing 0.25 mM MgCl_2 and 0.1 M PAPP to make an assay mixture thermostated at 37 °C. After a fixed time (usually 10 min) for the enzymatic reaction, an aliquot (10 μl) of the assay mixture was syringed out and added into the basal solution in an electrolysis cell, the mixture was then stirred for a few seconds. The oxidation current at +0.15 V was measured. It has been shown that the detection limit of alkaline phosphatase by the present electrochemical method, when the basal solution containing $3.3 \times 10^{-6} \text{ M}$ diaphorase was used, was 1×10^{-7} units (equivalent to 4×10^{-19} moles) in a 50 μl assay

mixture. The sensitivity of the present method is comparable to that of the chemiluminescent method.¹²

The experimental results show that the present method of the amperometric detection of alkaline phosphatase activities can be the basis for developing sensitive electrochemical enzyme immunoassays using alkaline phosphatase catalysis. This method also appears promising for enzyme immunoassays based on other enzyme catalysis, such as galactosidase catalysis. Also, other redox enzymes in the place of diaphorase, like glucose oxidase, as well as other redox substrates, are being investigated.

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