Notes

Rapid Measurement of Cholinesterase Activity Using an Amperometric Enzyme Electrode Based on Lipid-Modified Choline Oxidase

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Cholinesterases, acetylcholinesterase (AChE, EC 3.1.1.7) and pseudocholinesterase (PChE, EC 3.1.1.8), hydrolyze acetylcholine (and other esters in the case of PChE). The determination of cholinesterase activity is of diagnostic value in liver diseases, malignant tumors, asthma and tuberculosis.¹ Further, a decrease of the AChE activity in erythrocytes is often indicative of poisoning from organo-phosphorus pesticides.²

The use of an amperometric choline-sensing electrode based on choline oxidase (ChOD) is a convenient way for providing simple and selective assays of cholinesterases.³⁻⁶ The enzyme activity is determined from the electrode response as follows.⁷ When the 100% response time of the electrode upon the addition of choline is expressed as τ (min), and when the relationship between the steady-state current response, Δi , and the concentration of choline, c (mM), is given as $\Delta i = Ac$ (A is a constant), a linear current change with time, $\Delta i / \Delta t$, is obtained τ min after the addition of $a \text{ U l}^{-1}$ cholinesterase into an acetylcholine-containing test solution with a slope of $10^{-3} Aa$. That is, the enzyme activity can easily be determined by measuring the rate of the current change for $t \geq \tau$. The determination, however, requires a rather long time, *i.e.*, at least a few minutes^{3,5}, since τ at the conventional choline-sensing electrode is 1–2 min. An enzyme electrode that exhibits a rapid response to choline is required for quickening the measurement of cholinesterase activity.

Recently we have prepared amperometric enzyme electrodes for glucose^{8,9} and L-lactate¹⁰ by using lipid-modified enzymes. An electrode coated with a thin layer of the lipid-modified enzyme exhibited a fast current response (100% response, a few seconds) to the analyte.^{8,10} This paper deals with the preparation of a choline-sensing electrode based on lipid-modified ChOD and the use of the electrode for the assays of PChE. The PChE activity from 2 to 2000 U l^{-1} could be determined within 10 s.

Experimental

Chemicals

The enzyme used were ChOD (EC 1.1.3.17, from

Alcaligenes sp., Toyobo; Grade III, 16 U mg^{-1}), PChE (from horse serum, Sigma, St Louis, USA; C7512, 20 U mg^{-1}) and peroxidase (EC 1.11.1.7, from horseradish, Toyobo; Grade II, 250 U mg^{-1}). A lipid, *N*-(α -trimethylammonioacetyl)didodecyl-L-glutamate chloride [$(\text{C}_{12})_2\text{gluN}^+\text{Cl}^-$], was obtained from Sogo Pharmaceutical, and Nafion [5% (w/v) solution] from Aldrich (Milwaukee, USA). Choline chloride and acetylcholine chloride were purchased from Sigma. All other chemicals were of analytical grade and were used without further purification. Deionized, doubly distilled water was used throughout.

Preparation of choline-sensing electrode

ChOD modified with $(\text{C}_{12})_2\text{gluN}^+$ was prepared according to the literature.^{8,11} A buffer solution (2 ml, 0.02 M potassium acetate buffer plus 0.1 M KCl, pH 6) containing 25 mg ChOD was mixed with an aqueous dispersion (20 ml) of 200 mg of the lipid. After incubation of the mixture at 4°C for 24 h, the precipitate formed was collected by centrifugation, washed with water and lyophilized. A light yellow powder (*ca.* 100 mg) of the lipid-modified ChOD was obtained. The modified ChOD was soluble in organic solvents such as benzene.

The ChOD content in the modified enzyme was determined by measuring the adsorption of flavin adenine dinucleotide within ChOD in a benzene solution of the lyophilized product at 450 nm. The enzyme activity was measured spectrophotometrically by using a peroxidase-phenol-4-aminoantipyrine chromogenic system.¹² The dispersed medium used for the measuring ChOD activity of the modified enzyme was 0.1 M potassium phosphate buffer (pH 7.7, 25°C).

A glassy carbon electrode (Bioanalytical Systems, West Lafayette, USA) was polished with a 0.05 μm alumina slurry, rinsed with water and dried. Then the electrode was dipped into a benzene solution of the modified ChOD [2% (w/v)] and the solvent was allowed to evaporate at room temperature for 5 min. Finally, Nafion membrane coating was made by dip-coating the electrode in a 0.5% (w/v) Nafion solution, which was prepared by diluting the 5% solution as received with a mixture of 2-propanol [50% (v/v)] and water.¹³ The

electrode was allowed to dry at room temperature for 1 h. The thickness of the modified ChOD/Nafion layer was *ca.* 2 μm .

Measurement of choline and cholinesterase

A potentiostat (HA-502, Hokuto Denko) was used in a three-electrode configuration for amperometric measurements: the enzyme electrode, an Ag/AgCl reference electrode and a platinum auxiliary electrode were immersed in 10 ml of a test solution in a cylindrical cell. The test solutions used were 0.1 M potassium phosphate buffer (pH 7.7) for the measurement of choline and the same buffer containing 10 mM acetylcholine for that of PChE. The pH of the buffer was close to the optimal pH for ChOD¹⁴ and for PChE.¹ The concentration of acetylcholine was several times larger than the K_m -value of PChE for the substrate.¹⁵ The solution was saturated with air and stirred by a magnetic bar. The temperature of the solution was kept at $25.0 \pm 0.2^\circ\text{C}$. The potential of the enzyme electrode was set at 1.0 V vs. Ag/AgCl, which was positive enough for oxidizing the hydrogen peroxide produced through the ChOD reaction.⁸ The enzyme electrode was stored in 0.1 M potassium phosphate buffer (pH 7.7) at 4°C when not in use.

The PChE activity was measured spectrophotometrically by using a ChOD-peroxidase-phenol-4-aminoantipyrine chromogenic system.¹⁶

Results and Discussion

The ChOD content in the lyophilized powder of the modified enzyme was 30%. The ChOD activity of the modified enzyme was determined to be 5 U mg^{-1} solid, which corresponded to 16 U mg^{-1} ChOD. The ChOD activity of the unmodified enzyme was 16 U mg^{-1} . These results show that the enzyme activity does not decrease during the modification process and that enzyme substrates, choline and oxygen, permeate easily into the lipid layer to reach the active site of the enzyme. The enzyme activity retained in the modified ChOD/Nafion layer was *ca.* 50 mU cm^{-2} .

Figure 1 shows a current-time curve for the enzyme electrode for the successive additions of choline. The current increased immediately after the addition of choline and reached a steady state within 3 s. The 100% response time was as short as those for the glucose-sensing electrode based on $(\text{C}_{12})_2\text{gluN}^+$ -modified glucose oxidase⁸ and for the L-lactate-sensing one using lactate oxidase modified with the same lipid.¹⁰ No discernible current response was obtained on a ChOD-free, Nafion-coated electrode. This indicates that the current increase on the enzyme electrode is caused by the oxidation of hydrogen peroxide, which is generated through the ChOD-catalyzed reaction. The enzyme electrode gave a linear current response up to 0.4 mM choline [$\Delta i/c = 2.92 \times 10^{-6} \text{ A mM}^{-1}$ in this concentration region] and a significant increase in the response was

Fig. 1 Current-time curve for the enzyme electrode obtaining on increasing the choline concentration in 0.05 mM steps.

observed with an increase in the choline concentration in the range $0.4 - 2 \text{ mM}$. The detection limit for choline was $0.3 \mu\text{M}$, and the relative standard deviation for ten measurements of 0.1 mM choline was 1.4%.

From the characteristics of the present enzyme electrode as a choline sensor, $\tau = 3 \text{ s}$ and $\Delta i/c = 2.92 \times 10^{-6} \text{ A mM}^{-1}$, we can expect that the addition of $a \text{ U l}^{-1}$ PChE to the acetylcholine-containing test solution brings about a linear current increase with a slope of $4.87 \times 10^{-11} \text{ A A s}^{-1}$ ($= 2.92 \times 10^{-9} \text{ A A min}^{-1}$) in the time range $t \geq 3 \text{ s}$, as described above.⁷ Figure 2 shows the current-time curves for the enzyme electrode upon the addition of different activities of PChE. The PChE activities given in Fig. 2 are the values determined by spectrophotometry.¹⁶ As expected, the electrode current increased linearly with time from 3 s to at least 15 s: the time required for determining PChE activity was less than 10 s, which was much shorter than that at conventional choline-sensing electrodes, 1 – 2 min.^{3,5} The rate of the current change obtained in the linear region was proportional to the PChE activity up to 2000 U l^{-1} . The value of the current-increasing rate/PChE activity obtained from the experimental results (Fig. 2) was $4.85 \times 10^{-11} \text{ A l U}^{-1} \text{ s}^{-1}$, which was very close to the value expected from the choline response. For the determination of PChE, this means that only a calibration for choline is needed and that the calibration for PChE itself can be omitted. This would be useful in practice. The detection limit for PChE was 2 U l^{-1} , and the RSD for 10 measurements of 100 U l^{-1} PChE was 2.0%. The range of PChE activity that can be measured by the present electrode and the relative standard deviation were similar to those reported in the previous papers.^{3,5}

The PChE activity in normal serum was around 3000 U l^{-1} . The present enzyme electrode is therefore suitable for the determination of serum PChE after being

Fig. 2 Current-time curves for the enzyme electrode by addition of: (A) 500; (B) 200; (C) 100; (D) 50; (E) 20 U l⁻¹ PChE to an acetylcholine-containing test solution.

diluted around 100 times with the acetylcholine-containing test solution. Although the potential applied to the enzyme electrode (1 V vs. Ag/AgCl) is rather high, this would not bring about a serious error for measuring serum PChE activity. The use of the Nafion layer was effective in reducing the current response from electrochemical interferents in serum, such as ascorbic acid and uric acid.^{3,5} Further, such interferents are expected to cause no serious change in the rate of current change due to the PChE reaction.

The long-term stability of the enzyme electrode was examined by determining 0.1 mM choline 20 times a day for 5 weeks. The average value of the electrode response for the 20 measurements did not decrease in 4 weeks. The modified ChOD/Nafion-coated electrode thus showed a high stability.

The combination of lipid-modified ChOD with a thin

coating of Nafion polymer provided a choline-sensing electrode with high performance characteristics such as rapid response and high stability. The enzyme electrode was used for the rapid measurement of PChE. AChE can, of course, be determined by using the enzyme electrode.

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