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Optimal Immobilization of Penicillinase for Ion-selective Electrode

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Penicillin sensor was prepared by immobilizing penicillinase (Pcase) on H^+-selective carboxylated poly (vinyl chloride) (PVC-COOH) membrane or cellulose filter membrane. The immobilization techniques are as follows. Pcase was immobilized with GTH on H^+-selective PVC-COOH membrane or some amount of BSA was dropped on that membrane. Another method to make immobilization is to mix type I Pcase with GTH and drop on a cellulose filter membrane. According to immobilization techniques, there were some differences in response properties of enzyme electrodes, however, all electrodes responded to Pcase-resistant penicillin derivatives. Pcase immobilized on cellulose filter membrane with H^+-selective PVC membrane eletrode was more stable and more sensitive to penicillinase-resistant penicillin derivatives than any other immobilization techniques.

Key words : Immobilization, Penicillinase, H^+-selective membrane, Cellulose filter membrane

INTRODUCTION

Over the last three decades, a variety of potentiometric ion- and gas- selective membrane have been used as a detector of biosensors. Most of bioreagents such as enzyme, animal- or plant-tissue and microorganism convert a substrate into products that can be contacted with the tranceducer[1-9].

The principle of operation of the sensor is that the change of H^+ concentration arising from the enzymatic reaction (Fig. 1) at the surface of the polymeric membrane can be detected as a local pH change by the H^+-selective membrane electrode.

The major point of enzyme biosensors is how immobilization techniques could affect the response properties. When enzyme was immobilized on the sensing surface to form a gel layer, thickness of gel layer and enzyme-loading factor are critical to good performance of biosensors. Especially, Cha *et al.*[4] reported that a membrane combined with thicker enzyme-layer which was crosslinked with GTH show more stable potential than thin monolayer of enzyme did. In that paper, the thick enzyme-layer was expected to protect from the effect of magnetic stirring, causing rapid fluctuation of ion in the thin monolayer of the enzyme membrane. Actually, when pure penicillinase (type I) was immobilized, noise happened in steady-state. We studied on the several immobilization techniques of Pcase to improve the response properties. For example, Pcase was immobilized with GTH on H^+-selective PVC-COOH membrane or some amount of BSA was dropped on that membrane. Another method to make immobilization is to mix type I Pcase with GTH and drop on a cellulose filter membrane. This filter membrane was expected to act as a protection layer. We examined a stability and a sensitivity of those enzyme electrode to Pc-G and other penicillin derivatives.

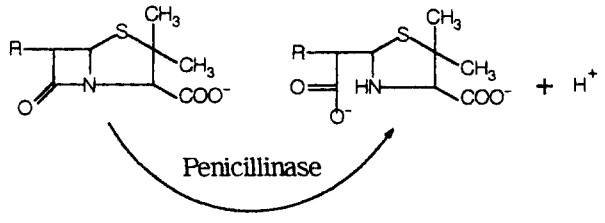

Fig. 1. Degradation reaction of penicillin with penicillinase.

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Until now, many penicillinase sensors have been reported[10-14]. However, no one referred to Pcase-resistant penicillin derivatives. In this paper, we examined on the optimal immobilization techniques of Pcase and the possibility for the determination of these penicillin derivatives.

MATERIALS AND METHODS

Reagents

Penicillin G-potassium salt (Pc-G), Pcase-resistant

penicillin derivatives (cloxacillin (Cx), dicloxacillin (Dx), methicillin (Mt), oxacillin (Ox), ticarcillin (Ti)) and Pcase (type I, type II from *Bacillus cereus*) were purchased from Sigma. Tetrahydrofuran (THF), PVC and PVC-COOH were perchased from Aldrich. H^+-selective ionophore (tridodecylamine, TDDA) and 2-nitrophenyloctyl ether (NPOE) were purchased from Fluka. Sodium tetraphenylborate (NaTpB) from Hayashi and GTH from TCI (Tokyo). All standard solution and buffer solution were prepared with deionized water. Type I Pcase solution (3.3 mg/100 μl) and type II Pcase solution (5 mg/ 100 l) were prepared by dissolving in 0.05 M phosphate buffer (pH 7.0), respectively. Working buffer solution was used as 3 mM phosphate buffer (pH 7.0) containing 0.1 M KCl. All standard solution of antibiotics were freshly prepared just before experiment.

Apparatus

Electrode body (IS-561, Phillips, Zürich) and single-junction Ag/AgCl electrode (Orion Co.) as external reference electrode were used. The potential of electrodes was measured with Orion pH/mV meter (Model 920A), which was connected to 386DX computer to store the potential obtained at every second intervals. Orion Rose combination glass electrode (Model 9102BN) was used to measure the pH of the buffer solution.

Ion-selective electrode fabrication

General procedure for the casting ion-selective membrane electrode was as follows; PVC or PVC-COOH (94 mg), NPOE (94 mg), TDDA (11 mg) and NaTpB (1 mg) were dissolved in 2 ml of THF. The solution was poured into a glass ring (i.d. 22 mm) fixed on glass plate (Fig. 2). The THF was evaporated for 2 days at room temperature. A disk membrane was cut by punch (i.d. 7 mm). After immobilization on the H^+-selective polymeric membrane, it was mounted on electrode body. Inner solution was used 0.02 M NaH_2PO_4/ 0.03 M Na_2HPO_4/0.015M NaCl solution.

Immobilization of enzyme

Four different methods of immobilization were employed. The procedure was as follows. The first method; 5 μl of Pcase (type I, II) and 5 μl of 2.5% GTH were dropped on H^+-selective PVC-COOH membrane and dried at 4°C for 1 day (Electrode I, II). The second method; the first membrane (type I Pcase) was added with various amount of BSA (Electrode III). The third method; the mixture of 5 μl of type I Pcase, 5 μl of 2.5% GTH and some amount of BSA was dropped on H^+-selective PVC-COOH membrane (Electrode IV). The fourth method; 5 μl of type I Pcase was mixed with 5 μl of 2.5% GTH solution and dropped on cellulose filter membrane (pore size: 0.45 m). After drying at 4°C for 1 day, this enzyme membrane overlapped with H^+-selective PVC membrane (Electrode V).

To remove any excess of glutaraldehyde, all enzyme electrodes were soaked in working buffer solution with magnetic stirring and stored in working buffer solution at 4°C when not in use.

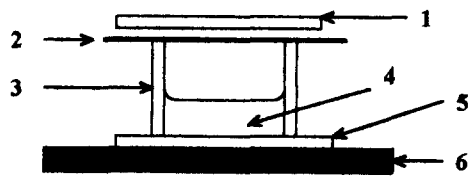

Fig. 2. Schematic diagram of casting membranes. 1. plane plate 2. filter membrane 3. glass ring (i.d.=22 mm) 4. THF solution 5. glass plate 6. desiccator plate

Measurements

All potentiometric measurements were carried out at room temperature. Electrodes were soaked in 10 ml of buffer solution until potential was stabilized as ±0.1 mV/min. Then small amount of Pc-G solution was added into that buffer solution. The potential was obtained when its rising rate was within +0.1 mV/ 30sec.

RESULTS AND DISCUSSION

Response properties of enzyme electrode

As shown in Fig. 3, the response of electrode I(A) was faster than other enzyme electrode, but its po-

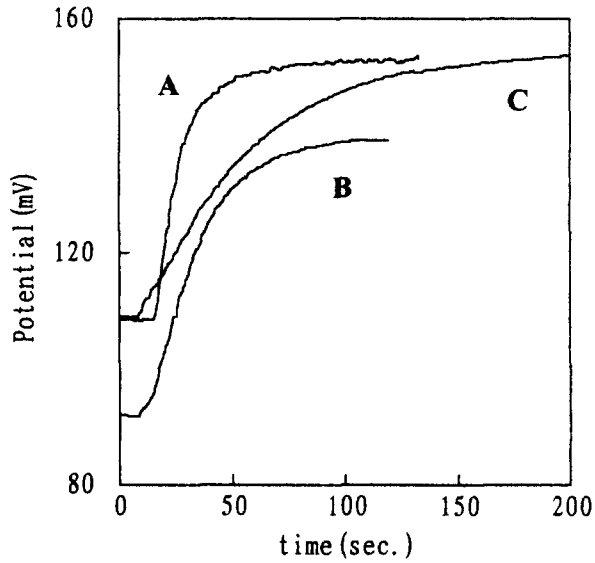

Fig. 3. Response curves of electrode I (A), electrode V (B) and electrode II (C) for 2 mM Pc-G.

tential was unstable. On the other hand, the response of filter-enzyme electrode(Electrode V, B) was slower than electrode I, but its potential was stable. Electrode II(C) showed stable potential with its long response time.

Protection effect of filter membrane

As shown in Fig. 3, protection layer such as a filter membrane is critical to the stability of steady-state potential. If there was no protection layer, it would be very difficult for monolayer of enzyme to protect the rapid fluctuations of the ion acitivity in steady-state. In this case, an added reagent could be diffused on enzyme surface rapidly and easily. The product ion could not be only detected but also diffused and fluctuated into buffer solution as fast as the reagent was diffused. Potential noise was found in steady-state (curve A). According to Cha *et al.*[4], thick layer could inhibit these rapid fluctuations. The effect of magnetic stirring was blocked by cellulose filter membrane (curve B). Although it has a long response time, immobilization on filter membrane has a merit of good stability.

Protection effect of BSA layer

BSA is a high molecular protein containing many carboxyl and amine group. As the alternative to filter membrane, a variety of BSA layer thickness were made to form a protection layer on the enzyme surface. In generally, the more amount of BSA was added, the thicker layer was formed. The more amount of BSA was added, the less its enzyme electrode responded to Pc-G (Fig. 4) and the more time was required to respond to Pc-G and to return back to the baseline potential. The degree of noise was less when the large amount of BSA was bound to the surface of enzyme layer. If BSA exist compactly on that layer, Pc-G could not react with Pcase efficiently because of hindrance of BSA layer. And the more amount of BSA is added, the less active site of Pcase is expected to be left because the rate of binding with BSA increases. In conclusion, addition of BSA on enzyme layer was not so good as immobilizing enzyme on cellulose filter membrane with GTH.

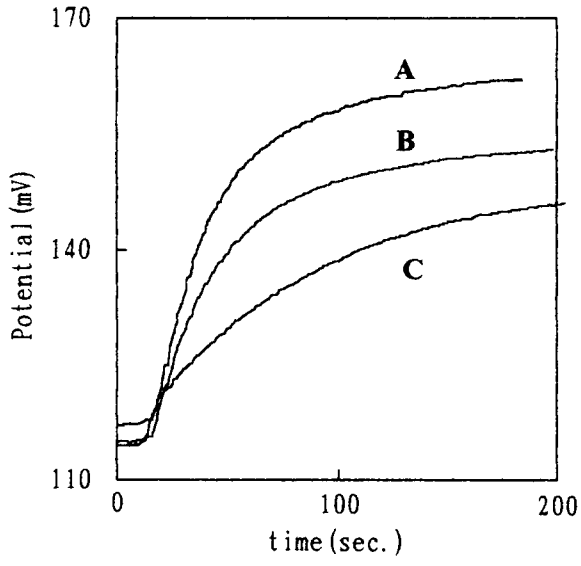

Fig. 4. Response curves of electrode III for 2 mM Pc-G. Added BSA solution was 1% (A), 2% (B) and 5% (C), respectively.

Protection effect of enzyme-BSA mixed layer

BSA is enough to increase the immobilizing efficiency of enzyme on the polymeric membrane. In this paper, type I Pcase was coimmobilized with 1% (A) and 2%(B) of BSA. In contrast to Fig. 4, the noise was decreased and the response time was not prolonged (Fig. 5). Noise of A, B curves was about ± 0.2~± 0.3 mV and ± 0.1~± 0.2 mV, respectively. However, the noise was increased after one day of measurement

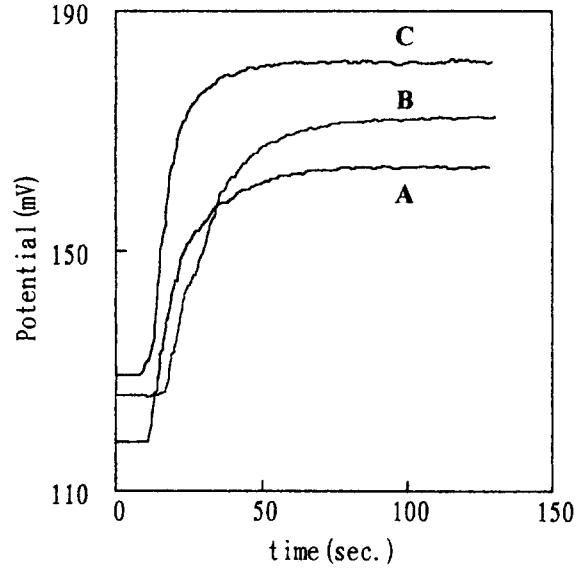

Fig. 5. Response curves of electrode IV for 2 mM Pc-G. Mixed BSA solution was 1% (A), 2% (B), respectively. Curve C was measured with electrode III (2% BSA) after one day measurement.

Table I. Potential differences of penicillin sensors to 2 mM solution of penicillin derivatives

Pc-deriv.	Potential difference (ΔmV)		
	Electrode	Electrode I	Electrode V
Pc-G	1.4	42.6	41.1
Ox	0.6	38.0	42.8
Ti	3.0	28.1	35.7
Cx	0.4	25.5	37.0
Dx	-0.6	21.1	32.6
Mt	0.8	27.7	37.2

Table II. Potential differences of electrode II and IV to 2 mM solution of penicillin derivatives

Pc-deriv.	Potential difference (ΔmV)	
	Electrode II	Electrode IV
Pc-G	45.7	52.0
Ox	26.0	35.8
Ti	24.0	31.0
Cx	5.3	13.1
Dx	4.2	8.0
Mt	24.2	30.1

(±0.3~±0.4 mV) (Fig. 5-C).

Potential Response to pcase-resistant antibiotics

Some antibiotics such as oxacillin, cloxacillin and dicloxacillin etc. are known to be resistant to some Pcase-producing strains because of bulk group around the α-carbon of the acylamino group, preventing Pcase from attaching to β-lactam ring. When experimented with filter-enzyme electrode, the response to these antibiotics was similar to Pc-G. As shown in table I and II, filter-enzyme electrode responded to all antibiotics better than any other enzyme electrodes did. These antibiotics may not be completely resistant to enzyme, and decomposed slowly. If there was no protection layer such as filter membrane, produced ion would be diffused into working buffer solution much faster than being detected to sensing device.

As a result, our filter-enzyme electrode was more useful than other immobilization techniques in terms of stability and sensitivity. It could detect not only Pc-G but also other Pcase-resistant penicillin derivatives.

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